Vol. 61, 1979

cta Haematologica

Founded 1948 by E. Meulengracht, K. Rohr and G. Rosenow
Continued by H. Lüdin (1960–1977)

Official Organ of the European Division of the International Society of Haematology
General Secretary J. W. Stewart, London

Karger Basel München Paris London New York Sydney



S. Karger AG 4011 Basel (Switzerland), Böcklin-Strasse 25
Printed in Switzerland by Buchdruckerei Zeitung + Basler Nachrichten AG Basel

Contents Vol. 61, 1979

No. 1

trlca. 5
er.s

...iginal Papers

Short Communications

No. 6

Original Papers

Correspondence

Publisher's Note

Acta haemat. 61: 1 (1979)

To Our Readers

Beginning with this volume *Acta Haematologica* is published in a two-column format, a space conserving design which permits the inclusion of more material in fewer pages. Although these volumes may feel slimmer than in the past, their actual contents have expanded.

We hope that this new format renders *Acta Haematologica* easier for you to read and handle and that you will appreciate these qualities as much as you will benefit from the increased amount of material presented.

Authors may consider that due to the two-column presentation the number of printed pages free of charge is now six (approximately 12 type-written pages).

During 1978 a number of additional referees assisted the Editorial Board with reviews of the submitted papers. The editors would like to thank the following colleagues:

H. Aebi
P. Auf der Maur
M. Baggiolini
E. A. Beck
H. Benöhr
A. Blumberg
K. Bürki
R. Bütler
J. P. Colombo
K. Deubelbeiss
F. Duckert
H. Fleisch
W. von Fliedner
M. Furlan
L. Garby
A. Gerber
J. Gmür
E. Gluckman
H. Graf
H. Heimpel
M. Hess
W. Hitzig
H. J. Huser
H. Isliker
M. Jeannet
H. U. Keller
H. Kummer
G. Martz
P. Massini
A. Morell
M. Neiger
E. Sauter
J. Seidel
R. Sonntag
B. Speck
G. A. Spengler
M. Stäubli
O. Tönz
L. Tschopp
O. N. Ulutin
H. P. Wagner
K. Winterhalter
P. Zahler
M. Zier
A. Zimmermann

Original Papers

Acta haemat. *61* 2-9 (1979)

Thrombocytopenic Episodes in Patients with Well-Functioning Renal Allografts

Inverse Relationship between Platelet Count and Platelet Size Pointing to Intermittent Platelet Destruction[1]

Th F Landis A von Felten and H Berchtold

Departments of Internal Medicine and Biostatistics, University of Zurich, Zurich

Key Words. Thrombocytopenia Large platelets Renal allotransplantation Platelet autoantibodies

Abstract. 20 out of 50 patients with well functioning renal allograft displayed at least one platelet count below $110 \times 10^9/l$ (mean –2 SD of controls) For estimation of platelet production during thrombocytopenic episodes, the percentage of large platelets in the peripheral blood was determined which revealed an inverse relationship ($p < 0.01$) to the platelet count, indicating that these thrombocytopenias were due to increased platelet consumption. Immunosuppressive treatment as well as rejection processes could be excluded as major pathogenetic factors whereas anti platelet autoantibodies may contribute to this phenomenon.

Preliminary observations revealed that renal allografted patients who were in excellent clinical conditions with respect to both renal function and other possible complications were occasionally exhibiting low platelet counts for which no reason could be established. Therefore, this study was designed in order to determine the incidence of such thrombocytopenic episodes and to characterize the underlying mechanism as either an intermittent inhibition of platelet production or a transient increase of platelet consumption

[1] Supported in part by the Fritz Rohrer Fonds and the Julius Müller Fonds

Methods

Patients. 50 renal transplant patients (16 women, 34 men, average age 39 years) of the Renal Transplant Group (Departments of Surgery A and Internal Medicine) at the University Hospital of Zurich were included in this study They were selected on the basis of a clinical steady state and controlled as out-patients at intervals of 1 2 or 3 months (average 10 weeks). Time interval between transplantation of cadaver kidney allograft and onset of our study amounted to ? months in 3 patients, 3 months in 1 patient and over 6 months in the other patients (average 31 months). At least three blood samples (maximum 7 average 5) per individual were examined, totalling to 248 samples tested. 4 patients were splenectomized prior to transplantation and are considered separately In

28 patients, treatment with azathioprine was unchanged throughout the entire period of this study (18, 4 and 6 patients received 200, 175 and 150 mg azathioprine/day respectively). 18 patients were treated with variable doses of this drug (variability of the daily dosage was less than 100 mg in 11 patients, 100–150 mg in 7 patients).

Control Subjects. In order to establish the normal platelet count as well as the normal percentage of large platelets in our laboratory 45 healthy laboratory personnel or medical school students (18 women, 27 men) were examined. None of them had donated blood for 3 months prior to this test.

Laboratory Tests. Blood was collected from vein into plastic tube containing dry ethylenediaminetetraacetate. The tube was kept at room temperature and the slide smears were obtained within 1 h. The smears were stained with May-Grunwald-Giemsa stain. Platelet count was performed manually according to the method of *Brecher and Cronkit* [3]. Artifactual thrombocytopenia was excluded by checking the corresponding blood smears for platelet satellitism [15]. Antiplatelet autoantibodies were determined by means of the microcomplement fixation test [22] with the patients own thrombocytes used as antigen.

Platelet size was measured by a slightly modified version of the microplanimetric method described by *Gary et al.* [9] the stained blood smears were projected at magnification of 1,000 times on microscope screen (Glarex, Carl Zeiss, FRG) equipped with grid made up of squares 2.5 mm wide. Platelets overlapping these squares in at least one direction were considered as megathrombocytes. For determination of the percentage of large platelets, the number of megathrombocytes served as the numerator, the total platelets counted as the denominator. In order to reduce statistical errors the enumeration procedure was extended to 600 megathrombocytes.

Additional determinations performed simultaneously included haemoglobin, red cell count, mean red cell volume, white cell count as well as serum creatinine.

Statistical Analysis. Mean values of different parameters obtained from patients and controls were compared by use of the two-sample t test.

In order to detect in individual patients any relationship in the variability of paired parameters, correlation coefficients calculated in each patient from individual test values of parameter pairs were submitted to the Wilcoxon signed rank test. In addition, correlation coefficients of mean values of individual patients were determined for selected pairs of parameters.

Results

Of a total of *248* platelet counts obtained from 46 renal transplant patients during a period of clinical steady state (minimum 5 maximum 22, average 9.4 months of observation), 68 were above 190×10^9/l (i.e. mean of controls), 56 between 190 and 150×10^9/l (mean −1 SD) 75 between 150 and 110×10^9/l (mean −2 SD), 41 between 110 and 70×10^9/l (mean −3 SD) and 8 below 70×10^9/l. *Mean values* of the different haematological parameters are given in table I. Besides a significant reduction of the mean number of thrombocytes, a striking increase of platelet heterogeneity was found as expressed by a highly significant rise of the mean percentage of megathrombocytes as well as of their standard deviation, whereas the average white blood cell count did not differ from control values. In addition, there was a significant elevation of the mean red cell volume. Haemoglobin and serum creatinine varied within a moderate range, reflecting the stable clinical situation in this selected group of patients.

Calculation of the mean of absolute numbers of megathrombocytes (table II) clearly indicates that the higher percentage of large platelets at different levels of platelet counts was always caused by a true increase of megathrombocytes and not by a 'relative megathrombocytosis' only [11].

Furthermore, there was a remarkable difference among the various tests per

Table I. Haematological parameters[1] in patients after renal allotransplantation compared with control values

	Patients	Controls	p values[2]
PC, × 10⁹/l	160 ± 55 (248)[3]	190 ± 40 (45)	< 0.001
% M, %	28.5 ± 12.8 (248)	13.5 ± 5.9 (45)	< 0.001
Hb (men), g/dl	13.6 ± 1.4 (117)	15.5 ± 1.25[4]	< 0.001
Hb (women), g/dl	12.3 ± 1.6 (54)	14.0 ± 1.25	< 0.001
MCV fl	106.4 ± 10 (132)	89.0 ± 3	< 0.001
WBC, × 10⁹/l	6.8 ± 2.2 (239)	6.6 ± 1.5	< 0.3
SeCr μmol/l	131.7 ± 57.5 (246)	84.0 ± 15	< 0.001

PC = Platelet count %M = percentage megathrombocytes Hb = haemoglobin MCV = mean red cell volume WBC = white blood cell count SeCr = serum creatinine

1 Mean values ± 1 SD
2 From two-sample t test.
3 Number of samples.
4 Normal values [6].

Table II. Increase of absolute numbers of megathrombocytes in renal transplant patients

	Platelet counts, × 10⁹/l		
	150–190 (n = 55)[2]	110–150 (n = 74)	< 110 (n = 49)
Absolute numbers of megathrombocytes, × 10⁹/l	53.8 ± 20.4	40.1 ± 15.8	32.3 ± 14.6
p values[3]	< 0.001	< 0.001	< 0.01

1 Individual platelet counts below 190 × 10⁹/l (n = 178) of all 50 patients were divided into three groups according to the number of platelets obtained.
2 n = Number of samples in each group
3 From two-sample t test with absolute numbers of megathrombocytes in controls (25.7 ± 11.3 × 10⁹/l, n = 45).

formed throughout the course of this study as far as their *variability within individual patients* was concerned. Whereas haemoglobin, mean red cell volume, white blood cell count and serum creatinine varied only moderately both platelet parameters were fluctuating to a considerable extent: 28 patients were displaying at least once a platelet count below 150 × 10⁹/l (mean –1 SD of controls) 20 patients below 110 × 10⁹/l (mean –2 SD of controls) the percentage of megathrombocytes was above 19.4% (mean + 1 SD of controls) and 25.3% (mean + SD of controls) in 40 and 31 patients respectively. As a representative example, the variability of the two platelet parameters compared with that of mean red cell volumes is depicted in figure 1. Thereby special attention has to be drawn to the fact that the same phenomenon was to be observed in the 28 patients who received an absolutely constant azathioprine treatment.

With these findings in mind, detailed statistical analysis were undertaken in order to prove any relevant relationship between certain pairs of parameters with respect to their variability occurring in individual patients. The results of the Wilcoxon tests performed with correlation coefficients between platelet counts or per cent mega-

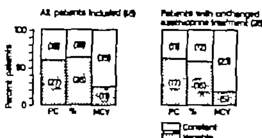


Fig. 1. Variability of platelet counts (PC), per cent megathrombocytes (%M), and mean red cell volumes (MCV) in individual patients. For parameter to be considered *variable* in patient, it had t cross the 'normal limit' in either direction t least once during the observation time. *Constant* parameters remained either constantly above or below this limit. 'Normal limits' were defined as mean control values minus 1 SD for platelets, plus 1 SD for megathrombocytes and mean red cell volumes respectively i.e. 150 × 10⁹/l PC, 19.4%M, and 92 fl MCV. Figures in parentheses denote number of patients.

thrombocytes, paired with all other haematological parameters, are summarized in table III. The only significant result was obtained within the pair 'platelet count/per cent megathrombocytes' where an inverse relationship could be demonstrated, i.e. low platelet numbers were associated with an increased morphological heterogeneity expressed as higher percentage of thrombocytes with a diameter over 2.5 μm. An identical correlation existed within the group of 28 patients who were under constant azathioprine treatment. Moreover this phenomenon was most pronounced in 19 patients in whom anti-platelet autoantibodies were detectable during our study

Special attention was rendered to a possible influence of azathioprine on size and amount of thrombocytes, but no relationship was to be found between this drug and

Table III. Wilcoxon signed rank test (p values) performed with correlation coefficients of paired parameters, obtained from individual patients

Pairs of parameters tested	All patients included (n = 46)	Patients with constant azathioprine *treatment* (n = 28)	Patients with anti-platelet utoantibodies (= 19)
PC/%M	< 0.01 inverse	< 0.01 inverse	< 0.001 inverse
PC/Hb	NS[1]	NS	NS
PC/MCV	NS	NS	NS
PC/WBC	NS	NS	NS
PC/SeCr	NS	NS	NS
%M/Hb	NS	NS	NS
%M/MCV	NS	NS	NS
%M/WBC	NS	NS	NS
%M/SeCr	NS	NS	NS

n = Number of patients
NS = Not significant (p > 0.1).

Table IV Statistical analysis with respect to correlation between azathioprine and other parameters

Pairs of parameters tested	Wilcoxon signed rank test (p values) patients with inconstant azathioprine treatment (= 18)[1]	Correlation coefficients of means (p values) all patients included (= 46)
Aza/PC	NS	(r = + 0.17) NS
Aza/%M	NS	(= + 0.12) NS
Aza/MCV	0.05 direct	(= + 0.23) NS
Aza/WBC	NS	(r = + 0.47) p < 0.001 direct

n = Number of patients.

By definition, only patients with inconstant azathioprine treatment could be included in Wilcoxon signed rank test.

Aza = dosage of azathioprine.

NS = Not significant (p > 0.1).

Table I. Haematological parameters[1] in patients after renal allotransplantation compared with control values

	Patients	Controls	p values[2]
PC, × 10⁹/l	160 ± 55 (248)[3]	190 ± 40 (45)	< 0.001
%M, %	28.5 ± 12.8 (248)	13.5 ± 5.9 (45)	< 0.001
Hb (men), g/dl	13 6 ± 1 4 (117)	15.5 ± 1.25[4]	< 0.001
Hb (women) g/dl	12.3 ± 1 6 (54)	14.0 ± 1.25	< 0.001
MCV fl	106.4 ± 10 (132)	89 0 ± 3	< 0.001
WBC, × 10⁹/l	6.8 ± 2.2 (239)	6.6 ± 1.5	< 0.3
SeCr μmol/l	131 7 ± 57.5 (?46)	84 0 ± 15	< 0.001

PC = Platelet count %M = percentage megathrombocytes Hb = haemoglobin MCV = mean red cell volume WBC = white blood cell count SeCr = serum creatinine.

1 Mean values ± 1 SD
2 From two-sample t test.
3 Number of samples.
4 Normal values [6].

Table II. Increase of absolute numbers of megathrombocytes in renal transplant patients

	Platelet counts, × 10⁹/l[1]		
	150–190 (n = 55)[2]	110–150 (n = 74)	< 110 (n = 49)
Absolute numbers of megathrombocytes, × 10⁹/l	53.8 ± 20.4	40.1 ± 15.8	32.3 ± 14 6
p values[3]	< 0.001	< 0.001	< 0.01

Individual platelet counts below 190 × 10⁹/l (n = 178) of all 50 patients were divided into three groups according to the number of platelets obtained.
2 n = Number of samples in each group.
3 From two-sample t test with absolute numbers of megathrombocytes in controls (25 7 ± 11.3 × 10⁹/l n = 45)

formed throughout the course of this study as far as their *variability within individual patients* was concerned. Whereas haemoglobin, mean red cell volume white blood cell count and serum creatinine varied only moderately both platelet parameters were fluctuating to a considerable extent: 28 patients were displaying at least once a platelet count below 150 × 10⁹/l (mean –1 SD of controls) 20 patients below 110 × 10⁹/l (mean –2 SD of controls) the percentage of megathrombocytes was above 19 4% (mean + 1 SD of controls) and 25.3% (mean + SD of controls) in 40 and 31 patients respectively As a representative example, the variability of the two platelet parameters compared with that of mean red cell volumes is depicted in figure 1 Thereby special attention has to be drawn to the fact that the same phenomenon was to be observed in the 28 patients who received an absolutely constant azathioprine treatment.

With these findings in mind, detailed statistical analysis were undertaken in order to prove any relevant relationship between certain pairs of parameters with respect to their variability occurring in individual patients. The results of the Wilcoxon tests performed with correlation coefficients between platelet counts or per cent mega-

relationship. Whether megathrombocytes are representing the population of young platelets which will progress with age to thrombocytes of smaller size [5 7 13 18 19 23] or whether heterogeneity of platelet size is rather reflecting the various mechanisms of enhanced megakaryocyte maturation [10 20 24–27] is still a question of debate.

We further substantiated the clinical usefulness of the 'megathrombocyte test by extensive statistical analysis, whereby special attention was paid to a possible influence of azathioprine in our patients. The elevation of the mean red cell volume in patients after renal allotransplantation is a well-known phenomenon which has been ascribed to this drug [16, 31] Azathioprine is supposed to cause megaloblastic anaemia through interfering in DNA synthesis [33] Since patients with megaloblastic anaemia on the basis of vitamin B_{12} or folate deficiency can develop a high percentage of megathrombocytes not correlated with an increased number of megakaryocytes [4 9] a similar effect of azathioprine on platelet size had to be considered despite the fact that no such data have been published so far *Schmidt et al* [31] have shown by serial measurements performed in individual patients that the mean red cell volume increases within 1 month after azathioprine treatment has been initiated and remains enlarged throughout the time of drug application. If azathioprine is exerting a similar effect on platelets, it would be reasonable to expect an equally constant enlargement of their volume However our results (fig. 1) are incompatible with this hypothesis In most of the patients, both platelet parameters showed a striking variability whereas mean red cell volumes were constantly increased. This fact was further confirmed by means of the Wilcoxon signed rank test, in which an inverse relationship between platelet count and percentage of megathrombocytes emerged as the only significant result with respect to these two parameters, i.e. a platelet fall being accompanied by a rise of megathrombocytes could be demonstrated in a significant majority of the patients. In cases of mild thrombocytopenia this inverse pattern has to be taken as proof of an increased peripheral platelet consumption. In no way could any relation be shown between platelets and azathioprine, not even in the group of patients who received this drug in variable amounts and in whom a dose-related increase of the mean red cell volume was found. Thus, any possible influence of azathioprine on platelet size, which may well exist, does not result in obliterating the episodes of morphological heterogeneity corresponding inversely with the platelet count.

Besides enhanced platelet destruction, hypersplenism is known to cause thrombocytopenia with increased heterogeneity of platelet size as well [9 17 24] The following facts are opposing this possibility (a) our patients did not display splenic enlargement to a clinically remarkable extent (b) splenic pooling would not lead to the episodic variability of platelet parameters observed in our cases [1 28] and, (c) 3 out of 4 patients who had been splenectomized prior to transplantation showed identical phases of platelet fall with concomitant rise of megathrombocytes.

Therefore, thrombocytopenic episodes which occur in a considerable number of renal transplant patients, have to be ascribed to a transient increase of platelet destruction. As far as their aetiology and clinical significance are concerned, little can be

either of the two platelet parameters (table IV) Since, by definition, 18 patients receiving variable amounts of azathioprine could be submitted to the Wilcoxon tests only an additional analysis was performed with the mean values of the respective parameters of all 46 patients Again no evidence of any connection between drug application and platelet parameters arose The direct relationship between white blood cell count and drug reflects the fact that the dosage is monitored according to the number of leukocytes.

Discussion

This study demonstrates that low platelet counts are to be observed with an unexpected high frequency in patients who have received kidney allotransplants, since 20 out of 50 patients showed at least one platelet count below 110 × 10⁹/l during a time of observation which lasted from 5 to 22 months. In addition, almost 60% of the patients were displaying in the same time period both normal as well as low values, characterizing thrombocytopenia mostly as a transient phenomenon.

The participation of platelets in hyperacute and acute rejection of heterografts and allografts in presensitized animals has been documented in a variety of animal models [30 32] Of special interest are the studies of *Ballantyne et al* [2] demonstrating that the level of circulating platelets was decreased during the rejection episode of first set and second-set skin allografts in rats. Also in human kidney allotransplantation, hyperacute and acute rejections are accompanied by an accumulation of thrombocytes in the rejected organ [14 21 29] The fact that a transient activation of platelet factor 3 could be found even during minor rejection episodes indicates that thrombocytes may be involved in such situations [12] In our patients, however intermittent decrease of circulating thrombocytes could hardly be caused by rejection processes, since this group of patients had been selected on the very base of a clinical steady state where no hint at a deterioration of renal function was detected throughout the time of observation hence additional factors causing thrombocytopenia were to postulate.

In pursuing this question we wanted to know whether these episodes of platelet decrease were due to reduced platelet production or increased platelet consumption. Two methods are generally used for quantifying platelet turnover i e determination of platelet survival from isotope disappearance curves after injection of ^{51}Cr labeled autologous thrombocytes [17] and the method of calculation of total platelet production from the megakaryocyte mass [10] We considered both of these procedures as an unreasonable demand on our group of renal transplant patients mainly for psychological motives beside the fact that daily collection of venous blood turned out to be extremely difficult in many patients. In this exceptional situation, we made use of the determination of the percentage of large thrombocytes ('megathrombocytes') in peripheral blood smears as described by *Garg et al* [9] This method is based on the fact that in a state of increased platelet production, a rise in platelet heterogeneity with a shift to large thrombocytes, can be observed. Thus, in situations of enhanced peripheral platelet consumption with consecutive rise of platelet production platelet counts and number of megathrombocytes will display an inverse

16 Klippel, J. H. and Decker J. L.: Relative macrocytosis in cyclophosphamide and azathioprine therapy. J. Am. med. Ass. 229: 180–181 (1974).
17 Kotilainen, M.: Platelet kinetics in normal subjects and in haematological disorders. With special reference to thrombocytopenia and the role of the spleen. Scand. J. Haemat., suppl. 5 pp. 12, 45 (1969).
18 McDonald, T. P.; Odell, T. T. and Gosslee, D. G.: Platelet size in relation to platelet age. Proc. Soc. exp. Biol. Med. 115: 684–689 (1964).
19 McDonald, T. P.; Clift, R., and Jones, J. B.: Canine cyclic hematopoiesis: platelet size and thrombopoietin level in relation to platelet count. Proc. Soc. exp. Biol. Med. 153: 424–428 (1976).
20 Minter, F. M. and Ingram, M.: Platelet volume: density relationships in normal and acutely bled dogs. Br. J. Haemat. 20: 55–68 (1971).
21 Mowbray, J. F. and Parjyananda, A.: Platelet thrombi in rejection of renal allografts. Thromb. Diath. haemorrh., suppl. 45 pp. 183–190 (1971).
22 Nymand, G.; Heron, I. and Jensen, K. G.: Micro complement fixation test for typing of platelets. A new method. Vox Sang. 20: 85–89 (1971).
23 O'Brien, J. R. and Jamieson, S.: A relationship between platelet volume and platelet number. Thromb. Diath. haemorrh. 31: 363–365 (1974).
24 Paulus, J. M.: Production et destruction des plaquettes sanguines (Masson, Paris 1974).
25 Paulus, J. M.: Platelet size in man. Blood 46: 321–336 (1975).
26 Penington, D. G.; Lee, N. L. Y.; Roxburgh, A. E., and McGready, J. R.: Platelet density and size: the interpretation of heterogeneity. Br. J. Haemat. 34: 365–376 (1976).
27 Penington, D. G.; Streatfield, K., and Roxburgh, A. E.: Megakaryocytes and the heterogeneity of circulating platelets. Br. J. Haemat. 34: 639–653 (1976).
28 Penny, R.; Rozenberg, M. C., and Firkin, B. G.: The splenic platelet pool. Blood 27: 1–16 (1966).
29 Porter, K. A.; Dossetor, J. B.; Marchioro, T. L.; Peart, W. S.; Rendall, J. M.; Starzl, T. E., and Terasaki, P. I.: Human renal transplants. I. Glomerular changes. Lab. Invest. 16: 153–181 (1967).
30 Rosenberg, J. C.; Hawkins, E., and Rector, F.: Mechanisms of immunological injury during antibody-mediated hyperacute rejection of renal heterografts. Transplantation 11: 151–157 (1971).
31 Schmidt, P.; Kopsa, H.; Pils, P.; Zazgornik, J. und Deutsch, E.: Makrozytose bei Patienten nach Nierentransplantation. Wien. klin. Wschr. 88: 532–535 (1976).
32 Sharma, H. M.; Rosensweig, J.; Chatterjee, S.; Moore, S., and de Champlain, M. L.: Platelets in hyperacute rejection of heterotopic cardiac allografts in presensitized dogs. Am. J. Path. 70: 155–164 (1973).
33 Stebbins, R.; Scott, J. and Herbert, V.: Drug-induced megaloblastic anemias. Semin. Hemat. 10: 235–251 (1973).

Received: April 11, 1978
Accepted: June 15, 1978

Dr. A. von Felten, Immunhämatologisches Labor, Departement für Innere Medizin, Universitätsspital, CH–8091 Zürich (Switzerland)

said for the time being. Certainly depression of thrombocytopoiesis, e.g. by azathioprine corresponding to its effect on leucocyte production can be ruled out. In addition decrease of platelet counts can be observed in these patients without any comprehensible deterioration of renal function. Therefore transient rejection processes cannot be considered as a major factor of causing platelet decrease and, certainly the determination of the number of peripheral thrombocytes will be of no help in monitoring rejection episodes. Anti platelet autoantibodies associated with lower platelet counts have recently been demonstrated after human renal allotransplantation [8] Since, in the present study episodes of decreased platelet counts were shown to be caused by enhanced platelet consumption and since patients with autoantibodies were displaying the most pronounced inverse relationship of platelet parameters, these autoantibodies may well contribute to thrombocytopenia. However further studies are required to entirely elucidate this noteworthy phenomenon of thrombocytopenic episodes occurring in patients after successful renal allotransplantation

Acknowledgements

We thank Dr *U Binswanger* Division of Nephrology Department of Internal Medicine, University Hospital, Zurich, for his kind permission to study these patients.

References

1 Aster R. H. Pooling of platelets in the spleen role in the pathogenesis of 'hypersplenic thrombocytopenia J clin. Invest. *45* 645–657 (1966)

2 Ballantyne, D L Coburn, R. J., Hawthorne, G A., and Nathan P Platelet levels during rat skin allograft rejection. Transplantation *13* 531–533 (1972).

3 Brecher G and Cronkite, E. P.. Morphology and enumeration of human blood platelets. J appl. Physiol. *3* 365–377 (1950).

4 Chatterji, A. K. and Lynch, E. C.. Circulating large platelets. New Engl. J Med. *284* 1440 (1971)

5 Corash, L., Tan, H., and Gralnick, H R.. Heterogeneity of human whole blood platelet subpopulations. I. Relationship between buoyant density cell volume, and ultrastructure. Blood *49* 71–87 (1977).

6 Dacie, J V and Lewis, S. M. Practical haematology· 5th ed., p. 12 (Churchill/Livingstone, Edinburgh 1975)

7 Detwiler T C. Odell, T T and McDonald, T P Platelet size, ATP content, and clot retraction in relation to platelet age. Am. J Physiol. *203* 107–110 (1962).

8 Felten, A. von, and Kuster J Antiplatelet autoantibodies with new specificities detected in patients after renal allotransplantation. Transplantation 22. 531–533 (1976).

9 Garg, S. K. Amorosi, E. L., and Karpatkin, S. Use of megathrombocyte as an index of megakaryocyte number New Engl. J Med. *284* 11–17 (1971).

10 Harker L. A. and Finch, C. A.. Thrombokinetics in man J clin. Invest. *48* 963–974 (1969)

11 Hillman, R. S. and Finch, C. A. The misused reticulocyte. Br J Haemat. *17* 313–314 (1969)

12 Kanfer A. Delarue, F and Languille, T Transient platelet factor 3 activation during human renal allograft rejection. Transplantation *18*. 78–81 (1974)

13 Karpatkin, S.. Human platelet senescence. A. Rev Med. *23* 101–128 (1972).

14 Kissmeyer Nielsen, F Olsen, S. Petersen, V P and Fjeldborg, O.. Hyperacute rejection of kidney allografts, associated with preexisting humoral antibodies against donor cells. Lancet *ii*. 662–665 (1966).

15 Kjeldsberg, C. R. and Swanson, J Platelet satellitism. Blood *43* 831–836 (1974).

Measurement of the Bactericidal Capacity of PMN. The killing test used is essentially that of *Quie et al.* [18]. Bacteria, leukocytes and opsonin were prepared in the same way. *Staphylococcus aureus* catalase (+) was used as the test organism. Bacteria were resuspended in saline to give an optical density of 0.300 at 650 nm in a Coleman photocalorimeter. This gave 2–3 × 10⁹ colony-forming units/ml. Leukocytes were suspended in Hanks' balanced salt solution (HBSS) with 0.1 / gelatin to give concentration of 2 × 10⁶ PMN/ml. Mononuclear contamination was generally about 5–15%. Sera were provided by 4 healthy persons and stored at –70 °C for no longer than 20 days. It was thawed immediately before use and diluted to 25% in HBSS. 0.5 ml PMN suspension, 0.1 ml bacteria suspension and 0.4 ml diluted serum were mixed. This provided about 2–3 bacteria/PMN and final concentration of 10%/ serum. Both at the beginning and after 140 min of incubation at 37 °C, 0.1 ml of this phagocytic mixture was diluted to allow the viable bacteria to be counted by the pour-plate technique. The percentage of viabl bacteria remaining after an incubation of 140 min was the measurement of bactericidal capacity.

Serum Opsonic Activity. This was estimated by comparing the total number of bacteria present in the patient's PMN in media containing patient's serum to the number obtained from the patient's PMN in media containing normal serum after 20 min incubation. At the end of the incubation period the tubes containing the phagocytic mixture were centrifuged at 250 *g* for 10 min and after the leukocyte pellets were washed twice in HBSS. A portion of the washed and resuspended leukocyte pellets (0.1 ml) were placed in distilled water for lysis and dilution as measure of leukocyte-associated bacteria [7].

MPO Activity. Measurement of MPO activity in PMN lysates depends on the disappearance of ultraviolet absorption band of uric acid at 290 nm. PMN lysates are prepared by homogenizing the isolated leukocytes in saline with glass-glass homogenizer. The specific activity is expressed as units/milligram of lysate protein [9].

Particle-Stimulated HMP Shunt Activity. Patient and control PMN (2 × 10⁷/ml) in 20% of the diluted serum mixture were stimulated by polystyrene latex particles whose mean diameter was 0.79 µm (Dow Chemical Co., Midland, Mich.), and $^{14}CO_2$ production from G-1-^{14}C (Radiochemical Centre, Amersham) was measured in the metabolic flask described by *Sabo and DiLuzio* (K-882351 Kontes Glass Co., Vineland, N.J.) according to *Sbarra and Karnovsky* [19]. All flasks were then incubated in an oscillating water bath at 37 °C for 60 min. A scintillation vial containing filter paper soaked with 10% KOH was then connected to the side arm of each flask. This trapped the $^{14}CO_2$ produced by the leukocytes during the experiment. The reaction was terminated by adding 1 ml of 62.5% (w/v) citric acid. Agitation continued for another 60 min to insure maximum absorption of ^{14}CO to the filter paper. This procedure was repeated without particles to determine the resting state activity.

NBT Dye Test. The quantitative NBT test was performed by the method of *Baehner and Nathan* [5].

Results

The mean hematological values are summarized in table I. These were significantly lower in the patient group when compared with our control group and with the criteria taken from WHO for the diagnosis of iron deficiency ($p < 0.001$).

The results of the NBT test, MPO activity and opsonic activity are given in table II. The NBT dye test in the resting phase and after stimulation was normal in all patients. MPO activity was found to be normal except in 1 case with decreased activity (0.02 U/mg). Opsonic activity was normal in all patients ($p > 0.05$).

The metabolic stimulation of HMP shunt was determined from the ratio of particle-stimulated $^{14}CO_2$ activity/resting $^{14}CO_2$ activity. This stimulation was found to be decreased in 29 cases and normal in 5 cases of the patient group. However the decrease was significant when compared with the control group ($p < 0.001$, table II).

Acta haemat. 61 10-14 (1979)

Myeloperoxidase Activity and Bactericidal Function of PMN in Iron Deficiency

Sevgi Yetgin Cigdem Altay Gonenc Cilv and Yahya Laleli

Departments of Pediatrics, Biochemistry and Radiology Children's Medical Center Hacettepe University Ankara

Key Words. Iron deficiency Bactericidal capacity Polymorphonuclear leukocyte Nitroblue tetrazolium test Hexose monophosphate shunt Myeloperoxidase

Abstract. In children with iron deficiency anemia, bactericidal capacity of polymorphonuclear leukocytes (PMN) and serum opsonic activity were studied. Nitroblue tetrazolium test (NBT) hexose monophosphate (HMP) shunt activation, and myeloperoxidase (MPO) activity of PMN of these cases were also examined. Bactericidal capacity and HMP shunt activation were found to be decreased in iron deficiency anemia ($p > 0.001$). MPO activity NBT test, and serum opsonic activity were found to be within normal limits. After $1^1/_2$ months of iron therapy there was an improvement in bactericidal capacity and it returned to a normal level after 3 months of therapy

It has been stated that individuals with iron deficiency anemias are more prone to bacterial infections [1 13 15 21] Recently impairment in cell mediated immunity and in bactericidal functions of polymorphonuclear neutrophils (PMN) have been demonstrated in the iron-deficient state [8 11 12, 14] Although deficiency in myeloperoxidase (MPO) activity has been suggested by *Arbeter et al* [2] the role of iron in bactericidal capacity has not been well explained. The present study was designed to measure bactericidal capacity nitroblue tetrazolium test (NBT dye test) hexose monophosphate (HMP) shunt activity MPO enzyme activity of PMN and serum opsonic activity

Materials and Methods

A total of 50 children (34 boys and 16 girls) with iron deficiency anemia were selected according to the criteria taken from WHO [16]. The mean age was 4 years, with a range of 6 months to 16 years. They were specifically selected, those iron-deficient patients who were malnourished or had infection at the time of examination were excluded The control group consisted of 25 children (16 boys and 9 girls) who were selected from the immunization clinic the mean age was 5 years. Both patient and control studies were made at the same time.

Hematologic Data. Routine hematological values were obtained by standard methods [22] Serum iron and total iron binding capacity were measured by the methods of *Fister* [10] and *Peter et al.* [17]

Discussion

Impairment of bactericidal capacity of PMN in iron-deficient subjects has been studied [8, 14] Our findings also clearly indicate that this abnormality can be expected to be present in all iron-deficient cases in childhood. The defective site of PMN causing bactericidal function abnormality is not known. A deficiency in MPO activity which has been shown by *Arbeter et al.* [2] is thought to be responsible for impairment of PMN bactericidal function. Cases of *Arbeter et al.* [2] had malnutrition in addition to iron deficiency anemia, and MPO deficiency of PMN has not been shown definitely in pure iron deficiency anemia [20] In our study MPO deficiency was found in only 1 patient, suggesting that, in iron deficiency anemia, MPO deficiency is a rare finding and should not be considered as a cause of deficient bactericidal function. Abnormality in HMP shunt activation which was present in 5 out of 34 patients was not expected to be present in the cases of MPO deficiency· our MPO-deficient patient had normal HMP shunt activity [3]

NBT test was found to be normal in our studies. Also, similar results were reported by *MacDougall et al.* [14], suggesting that the mechanism which is responsible for impairment in the killing capacity of PMN in iron deficiency anemia is different from that found in chronic granulomatous disease.

It is known that H_2O_2 degradation occurs by NADPH H+ as a cofactor which is reduced by the glutathione system in HMP shunt and stimulation of HMP shunt activation is due to NADPH H+ oxidation. It is expected that H_2O_2 degradation would be decreased in our cases because of insufficient HMP shunt stimulation [4] This finding may be explained by the inefficiency of the glutathione peroxidase system.

Some improvement in bactericidal capacity after 1 / months of iron therapy and complete improvement of this function after 3 months of therapy indicates that iron deficiency is responsible for deficient bactericidal capacity of PMN.

The absence of a severe infection in iron deficiency is probably related to the increased level of unsaturated transferrin and its bacteriostatic capacity [6] and/or to the limited duration of the deficiency state.

References

1 Andelman, H. M. and Sered, B. R.. Utilization of dietary iron by term infants. Am. J Dis. Child *11* 45–55 (1966).

2 Arbeter A., Acheverri, L. Franko, D; Munson, D and Velez, H.. Nutrition and infection. Fed. Proc. Fed. Am. Socs exp. Biol. *30*: 1421 1428 (1971).

3 Baehner R. L.: Disorders of leukocytes leading to recurrent infection. Pediat. Clins N Am. *19* 935–956 (1972).

4 Baehner R. L., Murrman, S. K., Davis, J *et al.* The role of superoxide anion and hydrogen peroxide in phagocytosis-associated oxidative metabolic reactions. J clin. Invest. *56*. 571 576 (1975).

5 Baehner R. L. and Nathan, D G.. Quantitative nitroblue tetrazolium test in chronic granulomatous disease. New Engl. J Med. *278* 1971 1976 (1968).

6 Caroline, L., Kozinn, P J Feldman, F Stiefel, F H., and Lichtman, H.. Infection and iron overload in thalassemia. Ann. N.Y Acad. Sci. *165*. 148–155 (1969).

7 Chandra, R. K.. Reduced bactericidal capacity of polymorphs in iron deficiency Archs Dis Childh. *48* 864–865 (1973).

8 Chandra, R. K. and Saraya, A. K.. Impaired immunocompetence associated with iron deficiency J Pediat. *86*. 899–904 (1975).

Table I. The means of hematological values

	Hb g/dl	PCV l/l	Serum iron μmol/l	Total iron binding cap μmol/l	Iron sat. %
Patients (50)	6.9 ± 1.8[1]	22.8 ± 5 6	4 9 ± 1.9	77 1 ± 16.9	6.5 ± 2.7
Control (25)	13 1 ± 1 0	38 4 ± 3.2	16.2 ± 1 8	52.5 ± 7 75	31 ± 4

Number of cases in parentheses.
[1] ± 1 SD

Table II. The mean of PMN functions, serum opsonic activity

Cases	Bactericidal capacity (% of remaining viable bacteria)	Serum opsonic activity No. of colony $\times 10^{-3}$ (10^6 PMN)	NBT test (ΔOD)	Metabolic stimulation[1]	MPO U/mg
Patient	28 4 ± 11 6 (50)	12.5 ± 4 1 (20)	0.08 ± 0 03 (20)	3 4 ± 2.0 (34)	0.09 ± 0.1 (22)
Control	5 1 ± 2.5 (25)	13 1 ± 5 0 (20)	0.08 ± 0.02 (14)	6.8 ± 2.1 (20)	0.07 ± 0.02 (20)
	$p < 0.001$	$p > 0.05$	$p > 0.05$	$p < 0.001$	$p > 0.05$

Figures in parentheses refer to the number of cases examined.
[1] Metabolic stimulation particle-stimulated $^{14}CO_2$ activity/resting $^{14}CO_2$ activity

Table III. Bactericidal capacity after iron therapy

Cases	Bactericidal capacity	p
Before therapy	31 ± 11 8 (11)	< 0.001
1 ½ months after therapy	19.8 ± 4.2 (11)	0.001
3 months after therapy	3 6 ± 1 6 (8)	> 0 05
Control	5 1 ± 2.5 (25)	

Figures in parentheses refer to the number of cases examined.

Bactericidal capacity of PMN was found to be decreased in all 50 patients examined, as shown in table II ($p < 0.001$) There is a positive correlation between the impairment of bactericidal capacity and the abnormality in shunt activation ($r = 0.6$)

Table III indicates the effect of iron therapy on bactericidal capacity in the patients whom we had the chance to follow up 1½ and 3 months after starting therapy The bactericidal capacity of PMN in 11 patients improved significantly ($p < 0.001$) but was still below the normal rates after 1½ months of iron therapy This function returned to normal in 8 patients examined after 3 months of iron treatment.

Acta haemat. 62: 15–26 (1979)

Evaluation of a Simplified Microchromatographic Technique for Hemoglobin A_2 Determination

A. Moors, J. Melis-Liekens, M. De Vlieger-Bensel, E. De Groof-Cornelis and G. Van Ros

Institute for Tropical Medicine, Laboratory of Hematology, Antwerpen

Key Words. Hemoglobin A_2 · Starch-block electrophoresis · Microchromatography · Beta-thalassemia trait · Iron deficiency anemia

Abstract. The simplified Hb A_2 determination based on microchromatography in Pasteur pipets filled with DEAE-cellulose with glycine-KCN-NaCl as developers [14] is compared with a reference Hb A_2 determination procedure based on starch-block electrophoresis. The utility of microchromatography as a routine Hb A_2 assay and as a screening method to detect β-thalassemia trait carriers and patients with iron deficiency anemia was investigated. Day-to-day variation of a control hemolysate and the correlation between the values obtained with the two methods and between determinations in duplicate on the same sample are given.

The mean values obtained with both methods for the different groups do not differ significantly but the standard deviations and the coefficients of variation observed by the microchromatography are generally higher. Microchromatography in Pasteur pipets tends to overestimate low and normal Hb A_2 concentrations and to underestimate high Hb A_2 concentrations. The results of microchromatography are more significant for the diagnosis when Hb A_2 concentrations are expressed in weight hemoglobin per volume of blood and not in percentages.

The microchromatographic procedure was recently marketed. The results obtained with the commercial columns were in good correlation with those obtained with starch-block electrophoresis, but commercial columns give a 18% overestimation of the Hb A_2 concentrations.

Introduction

The quantitation of the minor component of human hemoglobin, hemoglobin A_2 (Hb A_2) is very important for the diagnosis of thalassemia minor and to differentiate this trait from iron-deficiency anemia [1, 5]. Various other hematologic disorders are associated with abnormal percentages of Hb A_2 [2, 4, 15, 16]. There exist several groups of Hb A_2 measurement procedures: electrophoretical, chromatographical, immunologi-

9 Colowick, S. P., Kaplan, S. D Methods in enzymology vol. II (Academic Press, New York 1955).
10 Fister H. J Manual of standardized procedures for spectrophotometric chemistry (Standart Scientific Supply Co., New York 1950)
11 Higgs, J M. and Wells, R. S. Chronic mucocutaneous candidiasis associated abnormalities of iron metabolism. Br J Derm. *86* suppl 8, pp 88–102 (1972).
12 Joynson, D H. M., Jacobs, A. Walker D M and Dolby A. E., Defect of cell mediated immunity in patients with iron deficiency anemia. Lancet *2* 1058–1059 (1972)
13 Lahey M. E., Iron deficiency anemia. Pediat. Clins N Am. *4* 481–496 (1957)
14 MacDougall, L. G Anderson, R., McNab, G M., and Katz, J The immune response in iron deficiency children impaired cellular defense mechanism with altered humoral components. J Pediat. *86*. 833–843 (1975)
15 Mackay H. M. Anemia in infancy: its prevalence and prevention. Archs Dis. Childh. *3* 117–147 (1928).
16 WHO: Nutritional anemias. WHO tech. Rep. Ser *503* 29 (1972)
17 Peter S. T., Giovanniello, T J., Apt, L., and Ross, J F.. A new method for the determination of serum iron binding capacity J Lab clin. Med. *48* 274–279 (1956).
18 Quie, P G., White, J G Holmes, B., and Good, R. A.. *In vitro* bactericidal capacity of human polymorphonuclear leukocytes: diminished activity in chronic granulomatous disease of childhood. J clin. Invest. *46*. 668–679 (1967)
19 Sbarra, A. J and Karnovsky M. L.. The biochemical basis of phagocytosis I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. J biol. Chem. *234* 1355–1362 (1957).
20 Sagone, A. L. and Balcerzak, S. P.. Activity of iron containing enzymes in erythrocytes and granulocytes in thalassemia and iron deficiency Am. J med. Sci. *259* 350–357 (1970).
21 Shaw R. and Robertson, W O Anemia among hospitalized infants. Ohio med. J *60*: 45–51 (1964).
22 Wintrobe, M. M. Clinical hematology: 7th ed. (Lea & Febiger Philadelphia 1974).

Received March 20, 1978
Accepted July 1 1978

Associate Professor Sevgi Yetgin,
Department of Pediatrics,
Children's Medical Center
Hacettepe University Ankara (Turkey)

Acta haemat. 61: 15–26 (1979)

Evaluation of a Simplified Microchromatographic Technique for Hemoglobin A_2 Determination

A. Moors, J. Melis-Liekens, M. De Vlieger-Bensel, E. De Groof-Cornelis and G. Van Ros

Institute for Tropical Medicine, Laboratory of Hematology, Antwerpen

Key Words. Hemoglobin A_2 · Starch-block electrophoresis · Microchromatography · Beta-thalassemia trait · Iron deficiency anemia

Abstract. The simplified Hb A_2 determination based on microchromatography in Pasteur pipets filled with DEAE-cellulose with glycine-KCN-NaCl as developers [14] is compared with a reference Hb A_2 determination procedure based on starch-block electrophoresis. The utility of microchromatography as a routine Hb A_2 assay and as a screening method to detect β-thalassemia trait carriers and patients with iron deficiency anemia was investigated. Day-to-day variation of a control hemolysate and the correlation between the values obtained with the two methods and between determinations in duplicate on the same sample are given.

The mean values obtained with both methods for the different groups do not differ significantly but the standard deviations and the coefficients of variation observed by the microchromatography are generally higher. Microchromatography in Pasteur pipets tends to overestimate low and normal Hb A_2 concentrations and to underestimate high Hb A_2 concentrations. The results of microchromatography are more significant for the diagnosis when Hb A_2 concentrations are expressed in weight hemoglobin per volume of blood and not in percentages.

The microchromatographic procedure was recently marketed. The results obtained with the commercial columns were in good correlation with those obtained with starch-block electrophoresis, but commercial columns give a 18% overestimation of the Hb A_2 concentrations.

Introduction

The quantitation of the minor component of human hemoglobin, hemoglobin A_2 (Hb A_2) is very important for the diagnosis of thalassemia minor and to differentiate this trait from iron-deficiency anemia [1–5]. Various other hematologic disorders are associated with abnormal percentages of Hb A_2 [2, 4, 15, 16]. There exist several groups of Hb A_2 measurement procedures: electrophoretical, chromatographical, immunologi-

9 Colowick, S. P Kaplan, S. D., Methods in enzymology vol. II (Academic Press, New York 1955)
10 Fister H J., Manual of standardized procedures for spectrophotometric chemistry (Standart Scientific Supply Co., New York 1950)
11 Higgs, J M. and Wells, R. S., Chronic mucocutaneous candidiasis associated abnormalities of iron metabolism. Br J Derm. *86*, suppl. 8, pp. 88–102 (1972).
12 Joynson, D H. M Jacobs, A. Walker D M and Dolby A. E., Defect of cell mediated immunity in patients with iron deficiency anemia. Lancet 2 1058–1059 (1972)
13 Lahey M E., Iron deficiency anemia. Pediat. Clins N Am. *4* 481–496 (1957).
14 MacDougall, L. G Anderson, R., McNab, G M and Katz, J: The immune response in iron deficiency children. impaired cellular defense mechanism with altered humoral components. J Pediat. *86* 833–843 (1975)
15 Mackay H. M., Anemia in infancy: its prevalence and prevention. Archs Dis. Childh. *3* 117–147 (1928).
16 WHO: Nutritional anemias. WHO tech. Rep. Ser *503* 29 (1972).
17 Peter S. T Giovanniello, T J., Apt, L., and Ross, J F A new method for the determination of serum iron binding capacity J Lab. clin. Med. *48*. 274–279 (1956).
18 Quie, P G., White, J G Holmes, B Good, R. A., *In vitro* bactericidal capaci human polymorphonuclear leukocytes: ished activity in chronic granulomatous di of childhood. J clin. Invest. *46*. (1967)
19 Sbarra, A. J and Karnovsky M. L. biochemical basis of phagocytosis I. I changes during the ingestion of part polymorphonuclear leukocytes. J biol. *234* 1355–1362 (1957).
20 Sagone A. L. and Balcerzak, S. P., iron containing enzymes in erythrocytes granulocytes in thalassemia and iron cy Am. J med. Sci. *259* 350–357 (1970).
21 Shaw R. and Robertson, W O., A among hospitalized infants. Ohio med. 45–51 (1964)
22 Wintrobe, M. M Clinical hematology 7th (Lea & Febiger Philadelphia 1974)

Received. March 20, 1978
Accepted July 1 1978

Associate Professor Sevgi Yetgin,
Department of Pediatrics,
Children's Medical Center
Hacettepe University Ankara (Turkey)

al. [3]. Red blood cells were isolated by centrifugation, washed three times with 9 g/l NaCl and lysed in the usual way with distilled water and toluene. Clear hemolysates were then prepared by centrifugation and kept at 4 °C. Unless otherwise mentioned the analyses were done within 2 weeks after collection. A control hemolysate mixture was composed from hemolysates of healthy persons and repeatedly analyzed during 2 weeks.

Quantifications of hemoglobin A_2 were performed by microchromatography executed as described by *Huisman et al.* [14] and compared with the results obtained with starch-block electrophoresis according to *Van Ros* [26]. Additional results were obtained from 54 microchromatographic Hb A_2 determinations on 27 samples carried out according to the instructions given by the manufacturer of the comercial columns and compared with 54 Hb A_2 determinations on starch block. Working with the commercial columns, 20 µl hemolysate diluted with 180 µl developer were used instead of whole blood hemolyzed just before application as recommended by the manufacturer. Statistical calculations were performed according to the following equations:

The correlation coefficient =

$$\frac{\Sigma xy - \frac{\Sigma x \Sigma y}{n}}{\sqrt{\left(\Sigma x^2 - \frac{(\Sigma x)^2}{n}\right)\left(\Sigma y^2 - \frac{(\Sigma y)^2}{n}\right)}}$$

being the first Hb A_2 value, y the other and the number of determinations.

$$\text{The slope of the regression line} = \frac{\Sigma xy - \frac{\Sigma x \Sigma y}{n}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}$$

The intercept of the regression line with the ordinate $= \frac{\Sigma y - a\Sigma x}{n}$.

$$\text{The standard deviation (SD)} = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n-1}}$$

For comparisons where it was possible to calculate correlation coefficient t t value was derived $t = \frac{r}{\sqrt{1 - r^2}}\sqrt{n - 2}$.

For the other comparisons of means and standard deviations, a t-independent value was calculated as follows t =

$$\frac{m_1 - m_2}{\sqrt{\left[\frac{(n_1 - 1)(SD_1)^2 + (n_2 - 1)(SD_2)^2}{n_1 + n_2 - 2}\right]\left[\frac{1}{n_1} + \frac{1}{n_2}\right]}}$$

Results

(1) The day-to-day variation over 2 weeks based on repeated Hb A_2 determinations on the same normal control hemolysate is given in table I and compared with the sole corresponding figures reported in the literature.

A comparison between our Hb A_2 determinations and the determinations of *Schmidt* [23] is not entirely possible because his pool contained slow-moving hemoglobin variants and because he used columns of 0.5 × 20 cm instead of the short ones of 10 cm. For normal and low values with the reference method (2.61 and 2.00% Hb A_2), microchromatography gives higher values (2.8 and 2.31 %) whereas for the high value of 5% a lower percentage (4.76%) is obtained. When using microcolumns to determine Hb A_2 the standard deviation and the coefficient of variation are higher than those obtained with starch-block electrophoresis. Repeated Hb A_2 determinations on the hemolysates of 4 persons in order to estimate the reproducibility are given in table II.

(2) In order to compare the microchromatography with starch-block electrophoresis, determinations in duplicate of the Hb A_2 percentages of samples with A_2 concentrations ranging from 1.8 to 6% were performed.

The correlation coefficient r between the 19 determinations in blocks is 0.98, the

cal [7] and batch [18 19 21] methods. The first two mentioned procedures are the more generally used. The electrophoretic separation of Hb A_2, has been performed on several support media such as starch block, paper or cellulose acetate since the discovery of this hemoglobin by *Kunkel* and *Wallenius* in 1955 [17] The percentage of Hb A_2 is determined after electrophoresis either by eluting the hemoglobin fractions or by scanning the stained electrophoretic pattern, the densitometric approach however being less reliable [24]

Chromatographic procedures using the anion exchangers DEAE-cellulose and DEAE-Sephadex® in analytical columns of at least 20 × 0.9 cm were proposed to quantitate Hb A_2 [6 13] A good separation of Hb A_2 from other hemoglobins is also possible on cation-exchangers such as CM-cellulose [8 12] Since 1970 some cheaper microchromatographic procedures have been proposed or described [11] One of them is the simplified procedure for the determination of hemoglobin A_2 of *Huisman et al.* [14] using DEAE-cellulose as medium and Pasteur pipets as columns, but no statistical values were published about the correlation of the proposed technique with a recommended procedure. Nevertheless, the authors give three figures which indicate that the percentages obtained by their simplified method correlate more or less with another rapid microchromatographic A_2 determination published by *Efremov et al* [9] with the remark that the level of Hb A_2 by their method tends to be slightly lower Yet *Efremov's* method itself had not been thoroughly investigated [25] except that *Schmidt et al.* [23] determined the day to-day variation of this method using control hemolysates. The first results about normal values and standard deviations o tained with a modification of the method of *Huisman* were published for mal subjects and for β-thalassemia I zygotes by *Galanello et al* [10] *Schmidt al* [23] reported recently the day-to-d variation for 2 samples with slow hemoglobins using *Huisman's* method larger columns and *Schleider et al* have just published the mean values of A_2 in red cell hemolysates of normal and β-thalassemia heterozygotes by *Huisman's* method

An evaluation of *Huisman's* microchromatographic Hb A_2 determination urgent because this technique has been cently brought out on the market (Hb Quik Column procedure – Helena I tories, Beaumont, TX 77704 USA) therefore compared the values obtained the simplified chromatographic as it is described by *Huisman et al* [1 with those obtained by a starch-block el trophoretic technique [26] which is a ommended procedure for which statisti values are available.

Material and Methods

Blood was obtained from the following jects. 59 normal healthy adults, 20 patients iron-deficiency anemia, 57 β thalassemia trait riers and 68 subjects with various hematolog abnormalities including cases of malaria and heterozygotes. Every blood sample was studied standard laboratory procedures for the determi tion of hemoglobin concentration, number of red cells and leucocytes, red cell dices and blood film examination. Serum iron l and iron binding capacity were determined by automated method using bathophenanthroline reagent [27]. Hemoglobins were examined starch gel electrophoresis and hemoglobin F terminations were made by the method of *Betl*

al. [3]. Red blood cells were isolated by centrifugation, washed three times with 9 g/l NaCl and lysed in the usual way with distilled water and toluene. Clear hemolysates were then prepared by centrifugation and kept at 4 °C. Unless otherwise mentioned the analyses were done within 2 weeks after collection. A control hemolysate mixture was composed from hemolysates of healthy persons and repeatedly analyzed during 2 weeks.

Quantifications of hemoglobin A_2 were performed by microchromatography executed as described by *Huisman et al.* [14] and compared with the results obtained with starch-block electrophoresis according to *Van Ros* [26]. Additional results were obtained from 54 microchromatographic Hb A_2 determinations on 27 samples carried out according to the instructions given by the manufacturer of the comercial columns and compared with 54 Hb A_2 determinations on starch block. Working with the commercial columns, 20 µl hemolysate diluted with 180 µl developer were used instead of whole blood hemolyzed just before application as recommended by the manufacturer. Statistical calculations were performed according to the following equations.

The correlation coefficient =

$$\frac{\Sigma xy \quad \frac{\Sigma x \Sigma y}{}}{\sqrt{\left(\Sigma x^2 \quad \frac{(\Sigma x)^2}{}\right)\left(\Sigma y^2 \quad \frac{(\Sigma y)^2}{}\right)}}$$

being the first Hb A_2 value, y the other and the number of determinations

The slope of the regression line a = $\dfrac{\Sigma xy \quad \frac{\Sigma x \Sigma y}{}}{\Sigma \quad \frac{(\Sigma\ )^2}{n}}$

The intercept of the regression line fth the ordinate = $\dfrac{\Sigma y \quad a\Sigma x}{n}$

The standard deviation (SD) = $\sqrt{\dfrac{\Sigma \quad \frac{(\Sigma x)}{}}{\quad 1}}$

For comparisons where it was possible to calculate correlation coefficient at t value was derived $t = \dfrac{}{\sqrt{1 \quad r^2}}\sqrt{n \quad 2}$

For the other comparisons of means and standard deviations, a t-independent value was calculated as follows t =

$$\frac{m_1 - m_2}{\sqrt{\left[\dfrac{(n_1 - 1)(SD_1)^2 + (n_2 - 1)(SD_2)^2}{n_1 + n_2 - 2}\right]\left[\dfrac{1}{n_1} + \dfrac{1}{n_2}\right]}}$$

Results

(1) The day-to-day variation over 2 weeks based on repeated Hb A_2 determinations on the same normal control hemolysate is given in table I and compared with the sole corresponding figures reported in the literature.

A comparison between our Hb A_2 determinations and the determinations of *Schmidt* [23] is not entirely possible because his pool contained slow-moving hemoglobin variants and because he used columns of 0.5 × 20 cm instead of the short ones of 10 cm. For normal and low values with the reference method (2.61 and 2.00% Hb A_2), microchromatography gives higher values (2.8 and 2.31 /) whereas for the high value of 5% a lower percentage (4 76%) is obtained. When using microcolumns to determine Hb A_2 the standard deviation and the coefficient of variation are higher than those obtained with starch-block electrophoresis. Repeated Hb A_2 determinations on the hemolysates of 4 persons in order to estimate the reproducibility are given in table II

(2) In order to compare the microchromatography with starch-block electrophoresis, determinations in duplicate of the Hb A_2 percentages of samples with A_2 concentrations ranging from 1.8 to 6% were performed.

The correlation coefficient r between the 19 determinations in blocks is 0.98, the

cal [7] and batch [18 19 21] methods. The first two mentioned procedures are the more generally used The electrophoretic separa tion of Hb A_2 has been performed on several support media such as starch block paper or cellulose acetate since the discovery of this hemoglobin by *Kunkel* and *Wallenius* in 1955 [17] The percentage of Hb A_2 is de termined after electrophoresis either by elut ing the hemoglobin fractions or by scanning the stained electrophoretic pattern the den sitometric approach however being less reli able [24]

Chromatographic procedures using the anion exchangers DEAE-cellulose and DEAE-Sephadex® in analytical columns of at least 20 × 0.9 cm were proposed to quantitate Hb A_2 [6 13] A good separation of Hb A_2 from other hemoglobins is also possible on cation-exchangers such as CM-cellulose [8 12] Since 1970 some cheaper microchromatographic procedures have been proposed or described [11] One of them is the simplified procedure for the determination of hemoglobin A_2 of *Huis man et al.* [14] using DEAE-cellulose as medium and Pasteur pipets as columns, but no statistical values were published about the correlation of the proposed technique with a recommended procedure. Neverthe less, the authors give three figures which in dicate that the percentages obtained by their simplified method correlate more or less with another rapid microchromatographic A_2 determination published by *Efremov et al.* [9] with the remark that the level of Hb A_2 by their method tends to be slightly low er Yet *Efremov*'s method itself had not been thoroughly investigated [25] except that *Schmidt et al* [23] determined the day to-day variation of this method using con trol hemolysates. The first results about normal values and standard deviations ob tained with a modification of the simplifi method of *Huisman* were published for mal subjects and for β-thalassemia zygotes by *Galanello et al* [10] *Schmidt al* [23] reported recently the day to-d variation for 2 samples with slow hemoglobins using *Huisman*'s method larger columns and *Schleider et al* have just published the mean values of H A_2 in red cell hemolysates of normal adu and β-thalassemia heterozygotes by *Huisman*'s method

An evaluation of *Huisman*'s microchro matographic Hb A_2 determination urgent because this technique has been re cently brought out on the market ('Hb Quik Column procedure – Helena La tories Beaumont, TX 77704 USA). therefore compared the values obtained the simplified chromatographic as it is described by *Huisman et al* [1 with those obtained by a starch-block trophoretic technique [26] which is a rec ommended procedure for which stati values are available

Material and Methods

Blood was obtained from the following jects. 59 normal healthy adults, 70 patients w iron-deficiency anemia, 57 β thalassemia trait riers and 68 subjects with various hematological abnormalities including cases of malaria and heterozygotes. Every blood sample was studied standard laboratory procedures for the tion of hemoglobin concentration, hemat number of red cells and leucocytes, red cell in dices and blood film examination. Serum iron leve and iron binding capacity were determined by ar automated method using bathophenanthroline a reagent [27] Hemoglobins were examined by starch-gel electrophoresis and hemoglobin F de terminations were made by the method of *Betk et*

al. [3]. Red blood cells were isolated by centrifugation, washed three times with 9 g/l NaCl and lysed in the usual way with distilled water and toluene. Clear hemolysates were then prepared by centrifugation and kept at 4 °C. Unless otherwise mentioned the analyses were done within 2 weeks after collection. A control hemolysate mixture was composed from hemolysates of healthy persons and repeatedly analyzed during 2 weeks.

Quantifications of hemoglobin A_2 were performed by microchromatography executed as described by *Huisman et al.* [14] and compared with the results obtained with starch-block electrophoresis according to *Van Ros* [26]. Additional results were obtained from 54 microchromatographic Hb A_2 determinations on 27 samples carried out according to the instructions given by the manufacturer of the comercial columns and compared with 54 Hb A_2 determinations on starch block. Working with the commercial columns, 20 µl hemolysate diluted with 180 µl developer were used instead of whole blood hemolyzed just before application as recommended by the manufacturer. Statistical calculations were performed according to the following equations:

The correlation coefficient =

$$\frac{\Sigma xy \quad \frac{\Sigma x \Sigma y}{n}}{\sqrt{\left(\Sigma x^2 \quad \frac{(\Sigma x)^2}{}\right)\left(\Sigma y^2 \quad \frac{(\Sigma y)}{}\right)}}$$

being the first Hb A_2 value, y the other and the number of determinations

The slope of the regression line $= \dfrac{\Sigma xy - \dfrac{\Sigma x \Sigma y}{n}}{\Sigma \quad \dfrac{(\Delta x)^2}{}}$

The intercept of the regression line with the ordinate $= \dfrac{\Sigma y - s\Sigma}{}$

The standard deviation (SD) $= \sqrt{\dfrac{\Sigma x \quad \dfrac{(\Sigma x)}{n}}{1}}$

For comparisons here it was possible to calculate correlation coefficient at t value was derived $t = \dfrac{}{\sqrt{1 \quad r^2}}\sqrt{\quad 2}$

For the other comparisons of means and standard deviations, t-independent value was calculated as follows t =

$$\frac{m_1 - m_2}{\sqrt{\left[\frac{(n_1 - 1)(SD_1)^2 + (n_2 - 1)(SD_2)^2}{+ n_2 - 2}\right]\left[\frac{1}{n_1} + \frac{1}{n_2}\right]}}$$

Results

(1) The day-to-day variation over 2 weeks based on repeated Hb A_2 determinations on the same normal control hemolysate is given in table I and compared with the sole corresponding figures reported in the literature.

A comparison between our Hb A_2 determinations and the determinations of *Schmidt* [23] is not entirely possible because his pool contained slow-moving hemoglobin variants and because he used columns of 0.5 × 20 cm instead of the short ones of 10 cm. For normal and low values with the reference method (2.61 and 2.00%/ Hb A_2), microchromatography gives higher values (2.8 and 2.31%/) whereas for the high value of 5 / a lower percentage (4 76%/) is obtained. When using microcolumns to determine Hb A_2 the standard deviation and the coefficient of variation are higher than those obtained with starch-block electrophoresis. Repeated Hb A_2 determinations on the hemolysates of 4 persons in order to estimate the reproducibility are given in table II.

(2) In order to compare the microchromatography with starch-block electrophoresis, determinations in duplicate of the Hb A_2 percentages of samples with A_2 concentrations ranging from 1.8 to 6%/ were performed.

The correlation coefficient r between the 19 determinations in blocks is 0.98, the

Table I. Day-to-day variations of hemoglobin A_2 values for control hemolysates over a 2-week period compared with hemoglobin A_2 values obtained by *Schmidt et al.* [23] with a related technique

Technique	Microcolumn	Block	Adapted microcolumn[1] of *Schmidt*	
n	12	8	22	22
m, %	2.8	2.61	2.31	4 76
Range, %	2.3–3.2	2.4–2.8	2–2.8	4–5 4
SD %	0.24	0.13	0.23	0.35
CV %	8.56	4.98	9.96	7.37

n = Number of determinations, m = mean value, SD = standard deviation, CV = coefficient of variation.
[1] The initial Hb A_2 percentages of the pools of *Schmidt* (2 and 5 4) were determined after chromatography on analytical DEAE-Sephadex 0.9 × 60 cm column and recombination of the hemoglobin fractions in known amounts [20].

Table II. Reproducibility of Hb A_2 determination on DEAE-cellulose columns number of determinations, mean value, standard deviation, range and coefficient of variation are given for 2 hemolysates the results are compared with those of *Galanello et al.* [10], which are indicated by asterisks

Hemolysate	Normal subject	AS heterozygote	Normal subject*	β-Thalassemic heterozygote
n	6	4	5	5
m, %	2.19	3.54	2.40	5.33
Range, %	1.91–2.62	3 63–3 8	2.06–2.60	5 19–5 62
SD	0.24	0.17	0.23	0.16
CV	10.9	4.8	9 6	3 1

slope s of the regression line is 1 02, with an intersection with the ordinate very near the point 0 and the values of t is 31 This means that the linkage between two Hb A_2 determinations on the same sample is extremely strong. Hb A_2 quantitations made in duplicate on columns give as results if the values for Hb A_2 are given in percentages, $r = 0.83$ $s = 0.78$ $i = 0.58$ $t = 13.5$ for $n = 86$ determinations. For Hb A_2 values expressed in g/dl i becomes 0 042 g/dl It means that also between column determinations in duplicate there is a very good correlation but the lower t value indicates a less significant correlation than that obtained with blocks. Figure 1 gives the comparison between the results of the determinations made in duplicate on columns.

(3) The correlation between the values found by electrophoresis and the values obtained with the columns are given in tables III and IV

Tables III and IV indicate a good general correlation between block and column determinations of Hb A_2 values. Hb A_2 determinations in duplicate on columns are superfluous because the mean Hb A_2 value so found does not correlate better with the Hb A_2 determinations in block than Hb A_2 values found after only one chromatography

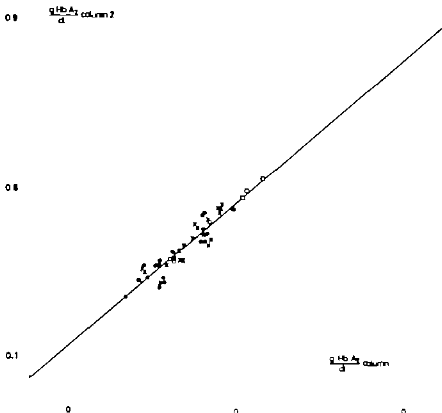


Fig. 1. A comparison of the Hb A_2 concentrations in g/dl blood obtained after microchromatographic determinations in duplicate. Analyses from 86 samples. 37 normal adults (×), 9 patients heterozygous for β-thalassemia trait (□), 6 persons with iron deficiency anemia (●) and 34 patients with other hematological disorders (O).

The mean Hb A_2 value for the different groups is nearly the same either on blocks or on columns. The standard deviations, however are generally higher for microchromatographic determinations.

Table V gives the probability and the t-independent value to distinguish between normals, thalassemic and sideropenic patients. Table V indicates that no clear-cut difference is observed between samples from normal persons and from patients with iron deficiency anemia if the Hb A_2 values are given in percent after column determination. To overcome this inconvenience the Hb A_2 values found after microchromatography have to be given in g Hb A_2/dl blood as proposed by *Ali and Schwertner* [1]

Figure 2 gives the general comparison between Hb A_2 determinations with both methods.

Table III. The mean and the standard deviation of Hb A_2 determinations and the correlation coefficient, the slope and the intercept of the regression line with the ordinate if column and block are compared for the four groups mentioned results obtained for Hb A_2 determinations in percentage of total hemoglobin concentration

Group	n	Method	m, %	SD %	r	s	i, %	t
Normal	55	block	2.72	0.31	0.21	0.20	2.09	3 6
		column	2.65	0.30				
β-Thalassemia	47	block	4.86	0 64	0.43	0.64	1 47	3.2
		column	4 61	0.95				
Iron deficiency anemia	17	block	2.53	0.31	0.1	0.13	-.15	0.39[1]
		column	2.49	0.42				
Various hematological disorders	58	block	2.62	0.39	0.39	0.46	1 49	3 15
		column	2.72	0.46				
All	177	block	3 11	1 0	0 82	0.80	0.55	20.5
		column	3.07	0.99				
Determinations in duplicate on the columns[2]	86	block	2.84	0.78	0.81	0.78	0.62	12.5
		column	2.84	0 75				

[1] Correlation between Hb A_2 values determined on block and on column for patients with iron deficiency not significant.

[2] Hb A_2 was determined twice on the column and once on the block.

(4) The results obtained with the commercially available columns were also compared with those of Hb A_2 determinations in starch-block.

The hemolysates of 4 normal adults, 3 persons with iron deficiency anemia, 10 persons heterozygous for β-thalassemia and 10 persons with other hematological disorders were analyzed. Hb A_2 of these samples was determined, twice on columns and twice on starch-blocks.

(a) The Hb A_2 concentration of 23 fresh samples from these persons was determined by starch-block electrophoresis then the samples were stored at −20 C during 1–9 months The second Hb A_2 determination on starch-block and the two Hb A_2 determinations on columns were performed within 14 days after the samples were thawed. The correlation between the double determinations for the blocks of this serie is r = 0 97 s = 0.95 i = 0 014 g/dl, t = 19 9 for n = 27 The linkage between two Hb A_2 determinations with block on the same material even spaced in time over 10 months is still very good and better than between Hb A_2 determinations in duplicate by microchromatography in Pasteur pipets (t = 13.5) Hb A_2 quantitation in duplicate on the same samples using commercial columns, however is also better than the microchromatography in duplicate in Pasteur pipets and comparable with the determinations made in block for this series. For commercial columns are r = 0 96 s = 0 91 i = 0 031 g/dl, t = 17 1 for n = 27

(b) The correlation between the Hb A_2 values in duplicate obtained by electrophoresis and those obtained by microchromatography on commercial columns are r = 0.94 s = 1 18 i = 7.42, t = 22.4 for n = 54 and r = 0 96 s = 1 19 i = 0 021

Table IV The values for m, SD s, l and t for the 4 groups studied results obtained for Hb A_2 determinations in g Hb/dl of blood

Group	n	Method	m, g/dl	SD g/dl	r	s	l, g/dl	t
Normal	55	block	0.387	0.055	0.53	0.58	0.151	4.5
		column	0 377	0.061				
β-Thalassemia	47	block	0.562	0.134	0.65	0.58	0.195	5.7
		column	0 525	0.120				
Iron deficiency anemia	17	block	0.269	0.053	0.62	0.82	0.044	3.1
		column	0.265	0.070				
Various hematological abnormalities	58	block	0.382	0 073	0.61	0.74	0 117	5.7
		column	0.399	0.087				
All	177	block	0.408	0.120	0.77	0.75	0.120	19.8
		column	0.404	0.116				
Determinations in duplicate on the columns	86	block	0.383	0.100	0.78	0.81	0.076	11.4
		column	0.386	0.104				

Hb A_2 was determined twice on the column and once on the block.

Table V Probability and t independent values to distinguish between normals β-thalassemic patients and sideropenic patients

Distinction between	Hb-A_2 values, %		Hb-A_2 values, g/dl	
	block	column	block	column
Normal and thalassemics	t = 21.86	t = 14 4	t = 8.85	t = 8.02
	0.001 > ~	0.001 > >	0.001 ~ > α	0.001 > ~
Normal and sideropenics	t = 2.21	t = 1 71	t = 7 76	t = 6.38
	0.05 >	0.1 >	0.001 > > α	0.001 > ~
Thalassemics and sideropenics	t = 12.01	t = 8.81	t = 8.73	t = 8.37
	0.001 > >	0.001 > >	0.001 > > α	0 001 > ~

Difference not significant.

g/dl, t = 17 1 for n = 27 These data are represented in figure 3

The correlations between determination in blocks, between determination in columns and be eter es in block this se a Hb

columns. The correlation coefficients between the values found by starch-block electrophoresis and the values obtained with commercial columns for the four groups of persons studied was between 0 77 and 0.97 It was noticed that the standard deviations of the column determinations tended to be somewhat higher than these of the determinations on blocks. Persons with β-thalasse-

Table III. The mean and the standard deviation of Hb A_2 determinations and the correlation coefficient, the slope and the intercept of the regression line with the ordinate if column and block are compared for the four groups mentioned results obtained for Hb A_2 determinations in percentage of total hemoglobin concentration

Group	n	Method	m, %	SD %	r	s	I %	t
Normal	55	block	2.72	0.31	0.21	0.20	2.09	3.6
		column	_.65	0.30				
β-Thalassemia	47	block	4.86	0.64	0.43	0.64	1 47	3.2
		column	4 61	0.95				
Iron deficiency anemia	17	block	2.53	0.31	0.1	0.13	2.15	0.39[1]
		column	_.49	0.42				
Various hematological disorders	58	block	2.62	0.39	0.39	0.46	1 49	3 15
		column	2.72	0 46				
All	177	block	3 11	1.0	0.82	0.80	0.55	20.5
		column	3.07	0.99				
Determinations in duplicate on the columns[2]	86	block	2.84	0.78	0.81	0.78	0.62	12.5
		column	2.84	0.75				

[1] Correlation between Hb A_2 values determined on block and on column for patients with iron deficiency not significant

[2] Hb A_2 was determined twice on the column and once on the block.

(4) The results obtained with the commercially available columns were also compared with those of Hb A_2 determinations in starch-block.

The hemolysates of 4 normal adults, 3 persons with iron deficiency anemia, 10 persons heterozygous for β-thalassemia and 10 persons with other hematological disorders were analyzed. Hb A_2 of these samples was determined, twice on columns and twice on starch-blocks.

(a) The Hb A_2 concentration of 23 fresh samples from these persons was determined by starch-block electrophoresis, then the samples were stored at –20 C during 1–9 months. The second Hb A_2 determination on starch-block and the two Hb A_2 determinations on columns were performed within 14 days after the samples were thawed. The correlation between the double determinations for the blocks of this serie is r = 0 97 s = 0.95 ı = 0 014 g/dl, t = 19.9 for n = 27 The linkage between two Hb A_2 determinations with block on the same material even spaced in time over 10 months is still very good and better than between Hb A_2 determinations in duplicate by microchromatography in Pasteur pipets (t = 13.5) Hb A_2 quantitation in duplicate on the same samples using commercial columns, however is also better than the microchromatography in duplicate in Pasteur pipets and comparable with the determinations made in block for this series. For commercial columns are r = 0 96 s = 0 91 i = 0 031 g/dl, t = 17 1 for n = 27

(b) The correlation between the Hb A_2 values in duplicate obtained by electrophoresis and those obtained by microchromatography on commercial columns are r = 0.94 s = 1 18, i = 7 42, t = 22.4 for n = 54 and r = 0.96 s = 1 19 i = 0 021

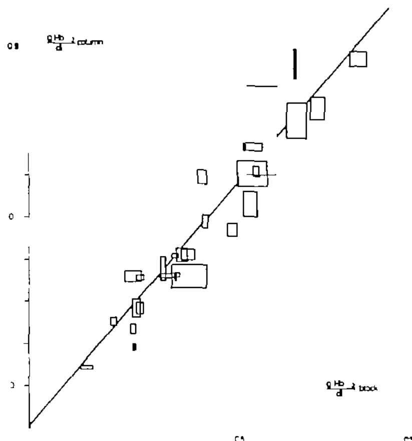


Fig. 3. Comparison of the Hb A_2 concentrations expressed in g/dl blood obtained with chromatography in commercial columns and with block electrophoresis. Double determinations on 27 samples. Each □ represents two Hb A_2 concentrations determined on column and two Hb A_2 concentrations determined after electrophoresis.

mia trait could be accurately detected with both methods till the same level of significance $\alpha < 0.001$

Discussion

The mean values obtained by the two methods of Hb A_2 determination for the different groups of subjects studied do not differ significantly the standard deviations, however calculated for the different groups are in general larger for microchromatography than for electrophoresis.

If one compares the Hb A_2 values obtained after block electrophoresis for normal adults with those of the patients with diseases which modify the Hb A_2 levels, the differences allow better to distinguish between them than the Hb A_2 levels obtained

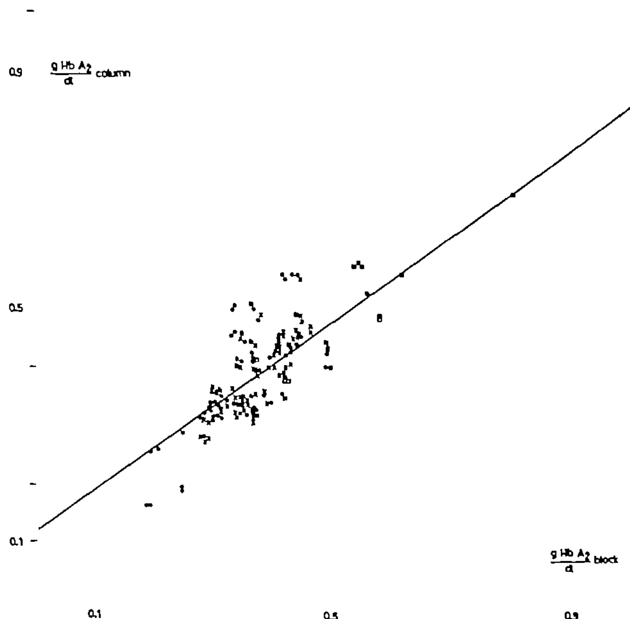


Fig. 2. Comparison of Hb A_2 concentrations in g/dl blood obtained with chromatography and with block electrophoresis. Analyses from 177 samples from 55 normal adults (×), 47 patients with β-thalassemia trait (□) (21 of these samples were stored at −20 °C from 1 month to 1 year) 17 persons with iron deficiency anemia (●) and 58 patients with other hematological disorders (○) The stored samples proved to be of reasonable quality after a new starch-block electrophoresis of the oldest ones.

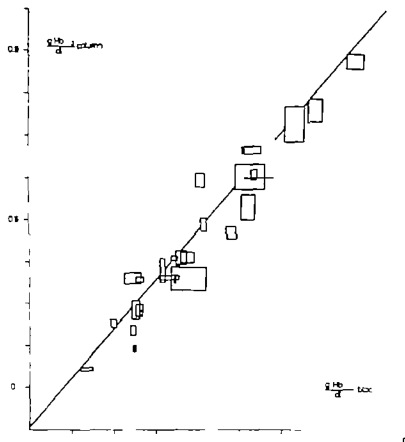


Fig. 3. Comparison of the Hb A_2 concentrations expressed in g/dl blood obtained with chromatography in commercial columns and with block electrophoresis. Double determinations on 27 samples. Each □ represents two Hb A_2 concentrations determined on column and two Hb A_2 concentrations determined after electrophoresis.

mia trait could be accurately detected with both methods till the same level of significance $\alpha < 0.001$

Discussion

The mean values obtained by the two methods of Hb A_2 determination for the different groups of subjects studied do not differ significantly the standard deviations, however calculated for the different groups are in general larger for microchromatography than for electrophoresis.

If one compares the Hb A_2 values obtained after block electrophoresis for normal adults with those of the patients with diseases which modify the Hb A_2 levels, the differences allow better to distinguish between them than the Hb A_2 levels obtained

after microchromatographic determinations (table V) From table V it is also clear that the microchromatographic Hb A_2 deter mination allows the differentiation of pa tients heterozygous for β-thalassemia from normal adults as already was stated by *Galanello et al* [10] but that cases of iron deficiency anemia are missed if the Hb A_2 values are expressed in percentages.

The intersection of the regression line with the ordinate and the slope of the re gression line indicate that microchroma tographic Hb A_2 determinations made in columns prepared in the laboratory overestimate low and normal Hb A_2 values and underestimate high Hb A_2 values this was also noted for the control hemolysates Hb A_2 values determined twice on commercial columns correlated well with each other and with the Hb A_2 values obtained with the blocks For commercial columns an overestimation of 18% was observed over the whole range of physiological Hb A_2 concentrations.

The differences between the results of starch-block electrophoresis and those of each of the two types of columns, either Pasteur pipets or commercial columns is difficult to explain. It probably depends on the procedure to determine the correct pH of the suspension used to fill the columns. Adjustment of the slurry to the right pH depends on the batch of DEAE-cellulose, on the precision of the pH measure ments on the manner how the slurry was stirred during pH measurement and on the response of the pH meter electrodes toward a stirred suspension of charged particles. The pH level is determined by running test chromatographies with fresh samples from Hb S heterozygotes at different pH's of the slurry from pH 7 6 till the pH at which Hb A_2 emerges in the first 3–4 ml and Hb S in the next 15–20 ml. Knowing that the slurry normally becomes more acid on standing which induces a rise in observed Hb A_2 val ues we set our pH so that Hb S just could been eluted in the next 20 ml. After 3–4 weeks the slurry is so deteriorated that it has to be discarded. Working with a slurry so prepared at a pH between 7.3 and pH 7 1 we observed for the correlations be tween Hb A_2 determinations in blocks and in Pasteur pipets the results given in table IV a correlation coefficient of 0 77 a slope of the regression line of 0 75 which means an underestimation of the Hb A_2 values of 25% for the low and normal Hb A_2 values, compensated by an intercept of 0 12 g/dl. The corresponding general correlation be tween Hb A_2 determinations in blocks and in commercial columns is characterized by a better correlation coefficient of 0.94 a slope of the regression line of 1 18 which means an overestimation of the Hb A_2 val ues of 18% and an intercept of –0 007 g/dl. The pH of 6 9 of the slurry of the commer cial columns explains the overestimation. The stability of that pH lies perhaps in the fact that the slurry was conserved in the tightly stopped columns till use, which was not the case for the slurry prepared in the laboratory this may explain the better cor relation coefficient observed with commer cial columns.

The slight difference observed between the two groups of interblock correlations is due to the use of frozen and thawed samples by the second interblock correlations deter mination The difference observed between the two groups of intercolumn correlation lies in the difference in executional schema that was followed for the determinations in duplicate on the same sample For the Hb

A_2 determinations in Pasteur pipets in duplicate the second Hb A_2 determination was done 1 week after the first with the same slurry after 1 week of conservation at 4 C or with a new adjusted slurry and with the same sample after 1 week conservated at 4 C. For the Hb A_2 determinations in commercial columns in duplicate, however the second Hb A_2 determination was performed the same day or the day thereafter

In general, one may state that the Hb A_2 determination with small columns gives good but slightly less reliable results than electrophoretic Hb A_2 determination on starch-block. The microchromatographic procedure may be very useful in conditions where it is impossible to use reference methods (many samples for screening, insufficient quantity of hemolysate, etc.). It is a very rapid and economic screening method.

References

1 Ali, A. M. and Schwertner E. Hemoglobin A_2 level. A proposed test for confirming the diagnosis of iron deficiency Am. J clin. Path. 63. 549–553 (1975).

2 Alperin, J B Dow P A., and Petteway M. B Hemoglobin A_2 levels in health and various hematologic disorders. Am. J clin. Path. 67 219–226 (1977).

3 Betke, K., Marti, H. R., and Schlicht, L. Estimation of small percentages of fetal haemoglobin. Nature, Lond. *184* 1877–1878 (1959).

4 Brabec, V Cejka, J and Vodrazka, Z. The A hemoglobin in hematological diseases. Clinica chim. Acta *28* 489–494 (1970).

5 Cartei, G Chisesi, T Cazzavillan, H., Battista, R.; Barbui, T and Dini, E. Relationship between Hb and Hb A_2 concentrations in beta-thalassemia trait and effect of iron deficiency anaemia. Biomed. Exp. 25. 282 284 (1976).

6 Chernoff, A. I A method for the quantitative determination of HgB A_2. Ann. N.Y Acad. Sci. *119* 557 560 (1964).

7 Chudwin, D. S. and Rucknagel, D. L. Immunologic quantification of hemoglobin F and A_2. Clinica chim. Acta *50* 413–418 (1974).

8 Dozy A. M. and Huisman, T H. J Studies on the heterogeneity of hemoglobin. XIV Chromatography of normal and abnormal human hemoglobin types on CM Sephadex. J Chromat. 40. 62 70 (1969).

9 Efremov G. D. Huisman, T H. J Bowman, K., Wrightstone, R. N and Schroeder W A. Microchromatography of hemoglobins. II. A rapid method for the determination of hemoglobin A_2. J Lab. clin. Med. *83* 657–664 (1974).

10 Galanello, R., Melis, M. A., Muroni, P and Cao, A. Quantitation of Hb A_2 with DE 52 Microchromatography in whole blood as screening test for β-thalassemia heterozygotes. Acta haemat. *57* 32 36 (1977).

11 Horton, B. F and Chernoff, A. L. Miniature column chromatography of hemoglobins. J Chromat. *47* 493 (1970).

12 Huisman, T H. J Chromatographic separation of hemoglobins A and C. The quantities of hemoglobin A_2 in patients with AC trait, CC disease and C-β-thalassemia. Clinica chim. Acta *40* 159–163 (1972).

13 Huisman, T H. J and Dozy A. M. Studies on the heterogeneity of hemoglobin. IX. The use of tris-(hydroxymethyl)-aminomethane-HCl. Buffers in the anion-exchange chromatography of hemoglobins. J Chromat. *19* 160–169 (1965).

14 Huisman, T H. J Schroeder W A., Brodie, A. N., Mayson, S M., and Jakway J Microchromatography of hemoglobins. III. A simplified procedure for the determination f hemoglobin A_2. J. Lab. clin. Med. *86*. 700–702 (1975).

15 Jonxis, J H. P and Huisman, T H. J A laboratory manual on abnormal haemoglobins (Blackwell, Oxford 1968).

16 Josephson, A. M., Masri, M. S Singer L., Dworkin, D and Singer K. Starch block electrophoresis of human hemoglobin solutions. II. Results in cord blood, thalassemia and other hematologic disorders comparison with Tiselius electrophoresis. Blood *13* 543–551 (1958).

17 Kunkel, H G. and Wallenius, G. New hemo-

globin in normal adult blood. Science, N Y *122*. 288 (1955)
18 Morin, L. G Rapid estimation of hemoglobin A_2 without chromatography or electrophoresis. Clin. Chem. *21* 1490–1492 (1975)
19 Morin, L. G Improved batch and column separation in the assay of hemoglobin A_2. Clin. Chem. *22* 2036–2038 (1976)
20 Moo-Penn, W F Jue, D L and Schmidt, R. M. Abnormal hemoglobin standards; in Abnormal hemoglobins and thalassemia diagnostic aspects (Academic Press, New York 1975).
21 Nastev D., Efremov G D and Petkov G A test tube method for quantitation of hemoglobin A_2 using DE 52 cellulose. Hemoglobin *1* 445–459 (1977).
22 Schleider C. T H., Mayson, S. M., and Huisman, T H. J.. Further modification of the micro-chromatographic determination of hemoglobin A_2. Hemoglobin *1* 503–504 (1977).
23 Schmidt, R. M. Brosious, E. M., and Wright, J M. Preparation and use of a quality control hemolysate for microchromatographic determi nation of hemoglobin A_2. Am. J clin. Path. *67* 215–218 (1977).
24 Schmidt, R. M. Rucknagel, D L., and Ne cheles, T F Comparison of methodologies for thalassemia screening by Hb A_2 quantitation. J Lab. clin. Med. *86* 873–882 (1975)
25 Sommer A. M Dujardin, P Audoly P and Chambor P Miniature chromatography of haemoglobin A_2 and heterozygous β thalassemia. Biomed. Exp *25* 307–310 (1976).
26 Van Ros, G Méthode de séparation et de purification des fractions de l'hémoglobine par électrophorèse en bloc d'amidon application au dosage de l'hémoglobine A_2. Annls Soc. belge Méd. Trop. *46*. 355–372 (1966).
27 Zak, B and Epstein, E. Automated determination of serum iron. Clin. Chem. *11* 641 (1965).

Received October 18, 1977
Accepted July 11 1978

Dr A. Moors,
Laboratory of Tropical Hematology
Institute of Tropical Medicine,
Nationalestraat 155, B-2000 Antwerpen (Belgium)

Acta haemat. 61 27–32 (1979)

Hematological and Hemoglobin Synthesis Studies in a Family with $\delta\beta$-Thalassemia Trait

J Pagnier K. P E. Amegnizin D Labie and M Hayat

Institut de Pathologie Moléculaire Inserm U-15, Laboratoire Associé au CNRS n° 85, Paris, and Groupe Hospitalier Paul-Brousse, Villejuif

Key Words. $\delta\beta$-Thalassemia Fetal Hemoglobin Thalassemia intermedia

Abstract. A Basque Spanish family with heterozygous $\delta\beta$-thalassemia is described. Patients with this anomaly usually present hematological findings observed in classical β-thalassemia, but clinical conditions and unbalanced chain synthesis are less severe. Our propositus, however presented clinical and biosynthetic data similar to those described in thalassemia intermedia. A family study was also performed.

$\delta\beta$-Thalassemia has been mostly encountered in Mediterranean [4 24 26, 27] and African populations [22], with only a few instances from other racial groups [14] This report describes a heterozygous form of the disease first suspected in a 9-year-old French girl of Basque Spanish and Italian parentage. Clinical and hematological data were similar to those described in thalassemia intermedia, but with 7% of fetal hemoglobin and a normal level of hemoglobin A_2. Biosynthetic studies were performed in order to confirm a defective synthesis of non-α-chains.

Material and Methods

were done by S Coulter was carried III, Helena ... was measured by alkali denaturation [3] and hemoglobin A_2 by elution and spectrophotometric quantitation at 415 nm after cellulose acetate electrophoresis [15]. The erythrocytic distribution of fetal hemoglobin was determined by the method of *Kleihauer et al.* [12]. The glycine:alanine ratio at the position 136 of the γ-chain was determined according to modification of the technique of *Schroeder et al.* [23]. Reticulocytes and bone marrow cells were incubated using ^{3}H-leucine as marker [13]. Whole-cell lysates were converted to globin and chains separated [6] after purification of the globin by Sephadex G-100 chromatography to remove nonheme protein [3]. Radioactivity was measured by liquid scintillation counting (Intertechnique SL 32).

Case Report

A 9-year-old girl presented in February 1976 with chronic anemia detected 2 years earlier Physical examination showed no abnormalities: spleen, liver and lymph nodes were not enlarged and there was no previous pathological history

globin in normal adult blood. Science, N Y *122* 288 (1955)

18 Morin, L. G Rapid estimation of hemoglobin A_2 without chromatography or electrophoresis. Clin. Chem. *21* 1490–1492 (1975)

19 Morin, L. G Improved batch and column separation in the assay of hemoglobin A_2. Clin. Chem. 22 2036–2038 (1976).

20 Moo-Penn, W F Jue, D L., and Schmidt, R. M. Abnormal hemoglobin standards in Abnormal hemoglobins and thalassemia diagnostic aspects (Academic Press, New York 1975).

21 Nastev D., Efremov G D and Petkov G., A test tube method for quantitation of hemoglobin A_2 using DE 52 cellulose. Hemoglobin *1* 445–459 (1977).

22 Schleider C. T H., Mayson, S. M., and Huisman, T H. J Further modification of the micro-chromatographic determination of hemoglobin A_2. Hemoglobin *1* 503–504 (1977)

23 Schmidt, R. M., Brosious, E. M., and Wright, J M. Preparation and use of a quality control hemolysate for microchromatographic determination of hemoglobin A_2. Am. J clin. Path. *67* 215–218 (1977)

24 Schmidt, R. M. Rucknagel, D L., and Necheles, T F.. Comparison of methodologies for thalassemia screening by Hb A_2 quantitation. J Lab. clin. Med. *86*. 873–882 (1975)

25 Soummer A. M., Dujardin, P., Audoly P and Chambor P Miniature chromatography of haemoglobin A_2 and heterozygous β thalassemia. Biomed. Exp *25* 307–310 (1976)

26 Van Ros, G.. Méthode de séparation et de purification des fractions de l'hémoglobine par électrophorèse en bloc d'amidon application au dosage de l'hémoglobine A_2. Annls Soc. belge Méd. Trop. *46* 355–372 (1966).

27 Zak, B. and Epstein, E.. Automated determination of serum iron. Clin. Chem. *11* 641 (1965).

Received. October 18, 1977
Accepted July 11 1978

Dr A. Moors,
Laboratory of Tropical Hematology
Institute of Tropical Medicine,
Nationalestraat 155 B-2000 Antwerpen (Belgium)

The biosynthesis of hemoglobin chains was studied using reticulocytes from each member of the family. An overall deficit of non-α-chains compared to α-chains was observed in the thalassemic subjects, the total non-α:α activity ratio ranging from 0.48 to 0.68 (fig. 2, table II). Synthesis was balanced in the reticulocytes of the unaffected members, i.e. the maternal grandfather (I 1), the father (II 1) and the eldest brother (III 1). In the two bone marrows examined (II 2, III-4) the synthesis was still unbalanced, but to a lesser degree than in the reticulocytes.

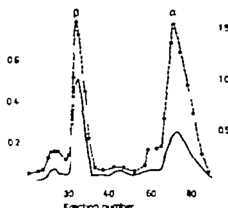


Fig. 2. Separation of the chains from a red blood cell lysate of the propositus (III-4). The cells are incubated with ³H-leucine. There is an excess of α-chain as compared with total non-α-radioactivity. — — = A_{280} nm; —— = cpm 10^{-3}/ml.

Discussion

Homozygous δβ-thalassemia is characterized by anemia and presence of only fetal hemoglobin. The heterozygous state shows the usual hematologic pattern of any thalassemia and characteristically a fetal hemoglobin ranging from 5 to 15%. As in other thalassemic syndromes, the fetal hemoglobin is unequally distributed in the red cells. There is a normal or slightly decreased percentage of hemoglobin A_2 [29]. This pattern

Table II. Total incorporation in non-α- and α-chains after incubation with ³H-leucine: the subjects with hematological thalassemic indices presented imbalanced chain synthesis

Subject	Total activity (cpm) and total activity ratio non-α/[illegible]					
	peripheral blood			bone marrow		
	non-α		non-α/[illegible]	non-α		non-α/[illegible]
I.1	17 721	19 617	0.90			
I.2	31,355	63,734	0.49			
II.1	68,390	63,940	1 07			
II.2	*11 774*	4,07?	0.49	2,355	3,861	0.61
III.1	20,452	22,690	0.90			
III.2	21,053	39,655	0.53			
III.3	33,092	52,644	0.63			
III.4	11,103	18,865	0.59	13,563	16,235	0.84

Hematological investigations revealed the following: hemoglobin 10.0 g/dl, red cell count 5.03 × 10[12]/l, reticulocytes 2.1%, packed cell volume 0.31, mean corpuscular volume 62 fl, mean corpuscular hemoglobin 19.8 pg, platelet count 250 × 10[9]/l and white cell count 4 1 × 10[9]/l (table I) The peripheral blood film was characterized by hypochromia and anisocytosis. The total serum bilirubin was 5 mg/l and serum iron level was 15.2 μM/l.

Her father is Italian and her mother of pure Spanish Basque origin. There were 4 sibs, 2 brothers also having hematological findings similar to the propositus.

Results

The hematological data are summarized in table I and the family tree is shown in figure 1 As well as the propositus (III–4), the maternal grandmother (I–2) the mother (II–2) and the 2 siblings (III–2 and III–3) presented peripheral blood film and red cell indices consistent with the diagnosis of thalassemia intermedia. The fetal hemoglobin level was elevated in the 3 9–8% range in all these subjects. Two other family members (II–1 and III–1) had slightly increased levels of fetal hemoglobin In all cases, the percentage of hemoglobin A_2 (2.4–3 0%) was within normal limits. The distribution of fetal hemoglobin in the red cells was heterogeneous The amino acid analysis of the C-terminal cyanogen bromide peptide of the γ-chain from the propositus demonstrated a glycine alanine ratio equal to 0 4 2.7

Table I. Hematological data on the family

Subject	Age years	Hb g/dl	PCV	RBC 10^{12}/l	Retic. %	MCV fl	MCH pg	MCHC g/dl	Serum Iron μM/l	Hb F %	Hb A_2 %
I.1	62	14.9	0.41	4.96	2.3	84	30.2	36.1	24.3	< 1	3.0
I.2	56	12.0	0.34	5 79	2.7	60	20.9	34.8		5 4	3.0
II 1	42	14.5	0.42	4.55	1.0	92	31.8	35.1	21.5	3 4	[illegible]4
II.2	37	10.9	0.34	5 67	1.3	61	19.2	32.5	19 7	3.9	3 0
III.1	15	13 7	0 40	4 79	0.5	84	28.6	34.9	23.2	2.6	2.8
III.2	13	10 4	0.31	5 45	1.5	58	19 1	33 7	20.6	8.0	2.5
III.3	11	10.7	0.32	5.38	1.5	61	19.8	33.5	22.4	6.6	2.9
III.4	9	10.0	0.31	5 03	2.1	62	19.8	32.4	15.2	6.9	2.5

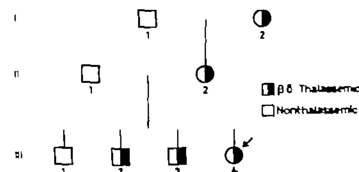


Fig. 1. Pedigree of δβ-thalassemic family

3 Betke, K., Marti, H. R., and Schlicht, L. Estimation of small percentages of foetal haemoglobin. Nature, Lond. *184* 1877–1878 (1959).

4 Brancati, C. and Baglioni, C. Homozygous $\beta\delta$-thalassaemia ($\beta\delta$-microcythaemia). Nature, Lond. *212*. 262–264 (1966).

5 Chalevelakis, G.; Clegg, J. B., and Weatherall, D. J. Imbalanced globin chain synthesis in heterozygous β-thalassemic bone-marrow. Proc. natn. Acad. Sci. USA *72*. 3853–3857 (1975).

6 Clegg, J. B., Naughton, M. A., and Weatherall, D. J. Abnormal human hemoglobins. Separation and characterization of the and β chains by chromatography and the determination of two variants: Hb Chesapeake and Hb J Bangkok. J. molec. Biol. *19* 91 108 (1966).

7 Clegg, J. B. and Weatherall, D. J. Hemoglobin synthesis during erythroid maturation in β-thalassemia. Nature new Biol. *240*: 190–192 (1972).

8 Forget, B. G. Benz, E. J. jr. Skoultchi, A.; Baglioni, C., and Housman, D. Absence of messenger RNA for beta globin chain in β^0-thalassemia. Nature, Lond. *247* 379–381 (1974).

9 Forget, B. G. Hillman, D. G., Lazarus, H., Barell, E. F. Benz, E. J. jr. Caskey C. T.; Huisman, T. H. J. Schroeder W. A., and Housman, D.: Absence of messenger RNA and gene DNA for β-globin chains in hereditary persistence of fetal hemoglobin. Cell 7 323–329 (1976).

10 Friedman, S., Schwartz, E., Ahern, E., and Ahern, V. Variations in globin chain synthesis in hereditary persistence of fetal haemoglobin. Br. J. Haemat. *32*. 357–364 (1976).

11 Kim, H. C., Marks, P. A., Rifkind, R. A., Maniatis, G. M., and Bank, A. Isolation and *in vitro* differentiation of human erythroid precursor cells. Blood *47* 767–776 (1976).

12 Kleihauer E., Braun, H. und Betke, K. Demonstration von fetalem Hämoglobin in den Erythrocyten eines Blutausstrichs. Klin. Wschr *35* 637–638 (1957).

13 Lingrel, J. B. and Borsook, H. A comparison of amino-acid incorporation into the hemoglobin and ribosomes of marrow erythroid cells and circulating reticulocytes of several anemic rabbits. Biochemistry 2. 309–320 (1963).

14 Mann, J. R., MacNeish, A. S., Bannister D. Clegg, J. B., Wood, W. G., and Weatherall, D. J. $\delta\beta$-thalassemia in Chinese family Br J Haemat. *25* 393–402 (1972).

15 Marengo-Rowe, A. J. Rapid electrophoresis and quantitation of hemoglobins on cellulose acetate. J. clin. Path. *18*. 790–792 (1965).

16 Natta, C., Banks, J. Niazi, G. Marks, P. A., and Bank, A. Decreased β globin mRNA activity in bone marrow cells in homozygous and heterozygous β-thalassemia. Nature new Biol. *244* 280–281 (1973)

17 Natta, C. L., Niazi, G. A., Ford, S., and Bank, A. Balanced globin chain synthesis in hereditary persistence of fetal hemoglobin. J. clin. Invest. *54* 433–438 (1974).

18 Nienhuis, A. W., Canfield, P. M., and Anderson, W. F. Hemoglobin messenger RNA from human bone marrow: isolation and translation in homozygous and heterozygous β-thalassemia. J. clin. Invest. *52*: 1735–1745 (1973).

19 Ottolenghi, S., Comi, P.; Giglioni, B., Tolstoshev P., Lanyon, W. G., Mitchell, G. J. Williamson, R., Russo, G., Musumeci, S. Schilliro G., Tsistrakis, G. A., Charache, S., Wood, W. G., Clegg, J. B., and Weatherall, D. J. $\delta\beta$-Thalassemia is due to gene deletion. Cell *9* 71–80 (1976).

20 Ottolenghi, S., Lanyon, W. G. Williamson, R., Weatherall, D. J. Clegg, J. B., and Pitcher C. S.: Human globin gene analysis for patient with $\beta^0/\delta\beta^0$-thalassemia. Proc. natn. Acad. Sci. USA *72*. 2294–2299 (1975)

21 Ramirez, F. O'Donnell, J. V. Marks, P. A., Bank, A., Musumeci, S. Schilliro, G., Pizzarelli, G., Russo, G., Luppis, B., and Gambino, R. Abnormal or absent β-mRNA in β^0 Ferrara and gene deletion in $\delta\beta$-thalassemia. Nature, Lond. *263* 471–475 (1976).

22 Ramot, B. Ben-Bassat, I.; Gafni, D., and Zaanoon, R. A family with three $\delta\beta$-thalassemia homozygotes. Blood *35* 158–165 (1970)

23 Schroeder W. A., Huisman, T. H. J. Shelton, J. R., Shelton, J. B. Kleihauer E. F. Dozy A. M., and Robberson, B. Evidence for multiple structural genes for the γ chain of human fetal hemoglobin. Proc. natn. Acad. Sci. USA *60* 537 544 (1968).

24 Silvestroni, E., Bianco, I., and Reitano, G. Three cases of homozygous $\beta\delta$-thalassemia

was observed in the 5 affected members of this family spread over three generations. Defective synthesis of non-α-chains was demonstrated in the reticulocytes and the diagnosis of δβ-thalassemia was indicated by the consistent finding of normal hemoglobin A_2 levels. The discrepancy between bone marrow and reticulocyte biosynthesis was not unexpected and has been noted by many others [7 11 16 18 31] The reason for the bone marrow ratio appearing to be nearer normal in non-α-thalassemias is still unknown. It has been suggested that there was proteolysis of excess α-chains [2, 7] or feedback regulation of the synthesis of α-chains [1] in nucleated cells.

The ratio at position 136 of the γ-chain gives a calculated proportion of the $^G\gamma$- to $^A\gamma$-chains of 2.3 (adult type) similar to that described in Greeks [26] The father (II 1) and brother (III 1) both had normal hematology and balanced chain synthesis. However they both had significantly raised fetal hemoglobin levels and the unequivocal presence of F cells on Kleihauer staining. These findings can be compared to high persistence of fetal hemoglobin (HPFH) of the heterogeneous Swiss type [28 30 32] The overall family findings are most readily explained on the basis of a δβ-thalassemia originating from the maternal grandmother with the addition of a Swiss type HPFH from the father This would explain the finding of a higher percentage of fetal hemoglobin in the thalassemic members in the third generation (III 2, 3 4) than in the first and second generations.

The patients with δβ-thalassemia have a variable degree of anemia less severe than the common Mediterranean β-thalassemia. The imbalanced globin chain production, when studied, is less important than in β-thalassemia [14] In this family hematological and clinical findings, as well as defective synthesis of non-α-chains, are more severe than in the previous described cases [4 14 22, 24 27] and clearly different from heterozygous HPFH [10 17 19 25 28]

Recent studies indicate that both δβ-thalassemia and HPFH are due to a deletion of β-chain gene and of all or part of δ-chain gene [8 9 19–21] The differences between them could be explained by the extent of the deletion involving control areas or not. Only a limited number of cases have been studied to date giving evidence of molecular heterogeneity but no data have been brought about the extent of the deletion in each case

This family is one more example of the heterogeneity of thalassemia and seems to illustrate a new type different by clinical and biological severity and by the European but not Mediterranean origin.

Acknowledgements

This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale' the 'Fondation pour la Recherche Médicale Française and the 'Sécurité Sociale

One of us is grateful to the 'Organisation Mondiale de la Santé for a fellowship

References

1 Bank, A. Gambino R. Ramirez, F Maniatis, G M.; Natta, C.; Kacian, D., Spiegelman, S., and Marks, P A. Regulation of globin synthesis in the thalassemias. Ann. N Y Acad. Sci. *241* 247–252 (1974)

2 Bank, A. and O'Donnel, J V.. Intracellular loss of free α chains in β-thalassemia. Nature, Lond. *222* 295–296 (1969).

Acta haemat. 61 33-38 (1979)

Hb Bart's and its Significance in the South African Negro

T S Pillszek

Department of Haematology The South African Institute for Medical Research, and School of Pathology University of the Witwatersrand, Johannesburg

Key Words. Haemoglobin Bart's α-Thalassaemia Neonatal microcytosis

Abstract. The haematological indices of cord bloods from 430 South African Negro babies were determined by electronic cell counting and their haemoglobin (Hb) patterns examined by alkaline cellulose acetate electrophoresis. A fast-moving, anodal band, identified as Hb Bart's, was found in 7 (1,6%) of the specimens, this being the lowest incidence of the variant yet found in an indigenous African population. The levels of Hb Bart's ranged from 1.3 to 5.5% of the haemoglobin. These findings were confirmed by alkaline-starch gel electrophoresis and at the same time absence of the slow-moving haemoglobin, Hb Constant Spring was established. *Subsequent follow-up of 4 of the infants at 4 months of postnatal* life showed that the abnormal component had disappeared. The babies with Hb Bart's had a marked microcytosis and low mean corpuscular haemoglobin levels whilst their parents showed no haematological or electrophoretic signs of α-thalassaemia. The significance of these findings is discussed in the light of previously reported studies on various Negro groups.

Haemoglobin (Hb) Bart's was probably first reported by *Fessas* and *Papaspyrou* [3] in a blood sample from a thalassaemic Greek infant and called Hb 'F and P'. The accepted name, however, was given to a new Hb variant, migrating as a fast anodal component on electrophoresis, found in an anaemic 1-month-old baby at St. Bartholomew's Hospital [1]. The red cell indices and morphology of both the infant and his parents suggested a thalassaemic picture. Nonetheless, electrophoretic findings in the parents showed normal levels of both Hb A_2 and F. Subsequent examination of this variant revealed that it consisted entirely of normal γ-globin chains arranged tetramerically [8].

Within 6 months of postnatal life, fetal Hb (HbF $\alpha_2\gamma_2$) is replaced by adult Hb (HbA, $\alpha_2\beta_2$) as the main Hb of the red cell. Usually this transition proceeds smoothly without any evidence of unbalanced globin-chain production. However trace amounts of Hb Bart's are found in most cord bloods

(or microcythaemia) with high haemoglobin F in a Sicilian family Acta haemat. *40* 220–229 (1968).
25 Sofroniadou, K. Wood, W G Nute, P E. and Stamatoyannopoulos, G Globin chain synthesis in the Greek type ($A\gamma$) of hereditary persistence of fetal hemoglobin. Br J Haemat. *29* 137–148 (1975).
26 Stamatoyannopoulos, G Schroeder W A. Huisman, T H J Shelton, J R., Shelton, J B., Apell, G and Bouver N., Nature of foetal haemoglobin in F-thalassaemia. Br J Haemat. *21* 633–642 (1971).
27 Tsistrakis, G A. Amarantos, S. P., and Konkouris, L. L. Homozygous $\beta\delta$-thalassaemia. Acta haemat. *51* 185–191 (1974).
28 Weatherall, D J Cartner R., Clegg, J B Wood, W G Macrae, J A and MacKenzie, A. A form of hereditary persistence of fetal haemoglobin characterized by uneven cellular distribution of haemoglobin F and the production of hemoglobins A and A_2 in homozygotes. Br J Haemat. *29* 205–220 (1975)
29 Weatherall, D J and Clegg, J B The thalassemia syndromes 2nd ed. (Blackwell, Oxford 1972).
30 Weatherall, D J and Clegg, J B Hereditary persistence of fetal haemoglobin. Br J Haemat. *29* 191–197 (1975)
31 Wood, W G and Stamatoyannopoulos, G Globin synthesis in fractionated normoblasts of β-thalassemia heterozygotes. J clin. Invest. *55* 567–579 (1975)
32 Wood, W G Weatherall, D J Clegg, J B. Hamblin, T J Edwards, J H., and Barlow A. M., Heterocellular persistence of fetal haemoglobin (heterocellular HPFH) and its interaction with β-thalassaemia. Br J Haemat. *36* 461–473 (1977)

Received. September 15 1977
Accepted. July 11 1978

Josée Pagnier Institut de Pathologie Moléculaire Inserm, CHU Cochin, 24, rue du faubourg Saint Jacques, F–75014 Paris (France)

Table II. Haematological mean values of South African Negro neonates with Hb Bart's and age-matched controls

	Controls (n = 77)		Infants with Hb Bart's (n = 7)	
	mean	SD	mean	SD
RBC, 10^6/µl	4.5	0.5	4.8	0.3
Hb g/dl	15.6	1.5	15.4	1.9
PCV %	48.0	5.0	45.6	4.8
MCV fl	108.4	5.6	95.0	4.9
MCH, pg	37.2	1.8	32.1	2.3
MCHC, %	32.5	1.1	33.6	1.2

Highly significant difference between means of the two groups ($p < 0.001$).

Table III. Haematological mean values of the parents of Negro infants with Hb Bart's

	Controls (n = 20)		Parents (n = 7)	
	mean	SD	mean	SD
RBC, 10^6/µl	5.4	0.3	5.2	0.1
Hb, g/dl	14.2	1.3	13.9	1.5
PCV %	43.9	1.8	42.6	2.3
MCV fl	85	2.8	82	1.2
MCH, pg	36.3	0.4	26.7	0.6
MCHC, %	30.9	0.5	32.8	0.6

abnormal component was confirmed to be Hb Bart's on electrophoresis in the 0.1 *M* phosphate buffer system and also by the presence of only γ-chains on hypermolar urea globin electrophoresis.

The 7 infants with Hb Bart's showed no gestational evidence of prematurity as evidenced by their birth weights. They had Hb Bart's levels ranging from 1.3 to 5.5 / and a marked microcytosis and lowering of mean corpuscular haemoglobin (MCH) levels when compared with a control group of Black full-term neonates (table II).

Statistical analysis of the results showed a significant negative correlation between the Hb Bart's level and the mean cell haemoglobin of these babies (fig. 1). Postnatal follow-up of 4 of them showed that the abnormal component had disappeared after 4 months although the abnormality of the red cell indices persisted.

The parents of these 4 infants were examined and showed no haematological abnormalities indicative of thalassaemia. One mother had to be excluded because of iron deficiency (table III). Electrophoretic studies were normal and there was no evidence of HbH inclusions on redox dye-stained slides.

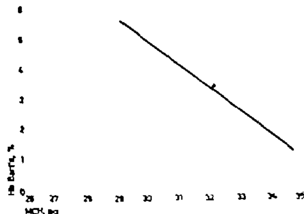


Fig. 1. Hb Bart's and MCH levels in South African Negro cord bloods.

possibly indicating a minor degree of imbalance of globin-chain synthesis during the switching-over process.

It is now well recognised that an increased blood level of Hb Bart's in the neonatal period is a sensitive indicator of α-thalassaemia in certain populations such as the Chinese, the Thais and Saudi Arabians. Other signs of α-thalassaemia, e.g. hydrops fetalis and HbH disease, have also been reported in these groups.

The position is less clear in Negroid populations with studies from America, Central and West Africa showing varying results although both qualitative and quantitative haemoglobinopathies are relatively common in these regions [11]. The possible presence of an α-thalassaemia gene in the indigenous populations of the Southern African Continent has been suggested by a report of a single case of HbH disease in a Black adult and of Hb Bart's in two Zambian neonates [2].

The purpose of this study was to determine the significance of these isolated reports and to see whether there would be any correlation between the finding of Hb Bart's and the possible presence of α-thalassaemia. Such information would cast further light on the understanding of the thalassaemia mutations in indigenous Black peoples.

The present survey was carried out in 1976 and 1977 and indicates the lowest reported incidence of elevated levels of Hb Bart's yet found in an indigenous Negroid population.

Materials and Methods

Unselected samples of placental cord blood were obtained from babies of mothers delivered in the Obstetrics Department of Baragwanath Hospital, Johannesburg. The blood was placed into ethylenediamine tetraacetic acid (EDTA) anticoagulant containers and red cell indices were determined using a Coulter Counter Model S [9]. Hb types were identified by cellulose-acetate electrophoresis in a glycine-EDTA-Borate buffer pH 9.2 on a Gelman Microtek electrophoretic chamber [5]. The Hb percentages were quantitated by elution spectrophotometry after electrophoresis in the same buffer using the semi-micro apparatus. The presence of a fast, anodal component was confirmed by starch-gel electrophoresis in a tris-EDTA-borate system, pH 8.5 [21].

Hb Bart's was further identified by cellulose-acetate electrophoresis in a 0.1 M phosphate buffer system, pH 7.0 and by globin electrophoresis in a 10 M urea-barbitone buffer system, pH 8.7 [17, 19].

Hb H preparations were made after incubation of equal volumes of blood and 1% brilliant cresyl blue at 37 °C for 3 h.

Results

Of 430 cord bloods studied 7 were found to have Hb Bart's (table I). No other abnormal Hb variants such as Hbs S, C or Constant Spring were detected by this cellulose acetate electrophoretic procedure. The

Table I. Haematological, electrophoretic and gestational data on 7 infants with Hb Bart's in their cord blood

Infant	Sex	Weight kg	Hb Bart's %	MCV fl	MCH pg
I.S.	F	2.80	5.5	88	28.2
C.M	F	3 10	2.3	96	32.8
C.M	M	3.05	3.5	91	32.2
N M.	F	3 70	4.9	94	31.0
F T	F	2.60	1.3	100	34.0
E.N	M	4 00	1.3	101	35.0
L.M	F	3.25	4.3	97	31.6

Table II. Haematological mean values of South African Negro neonates with Hb Bart's and age-matched controls

	Controls (n = 77)		Infants with Hb Bart' (n = 7)	
	mean	SD	mean	SD
RBC, $10^6/\mu l$	4.5	0.5	4.8	0.3
Hb, g/dl	15.6	1.5	15.4	1.9
PCV %	48.0	5.0	45.6	4.8
MCV fl	108.4	5.6	95.0	4.9
MCH, pg	37.[illegible]	1.8	32.1	2.3
MCHC, %	32.5	1.1	33.6	1.2

Highly significant difference between means of the two groups ($p < 0.001$).

Table III. Haematological mean values of the parents of Negro infants with Hb Bart's

	Controls (n = 20)		Parents (n = 7)	
	mean	SD	mean	SD
RBC, $10^6/\mu l$	5.4	0.3	5.2	0.1
Hb, g/dl	14.2	1.3	13.9	1.5
PCV %	45.9	1.8	42.6	2.3
MCV fl	85	2.8	82	1.2
MCH, pg	36.3	0.4	36.7	0.6
MCHC, %	30.9	0.5	32.8	0.6

abnormal component was confirmed to be Hb Bart's on electrophoresis in the 0.1 *M* phosphate buffer system and also by the presence of only γ-chains on hypermolar urea globin electrophoresis.

The 7 infants with Hb Bart's showed no gestational evidence of prematurity as evidenced by their birth weights. They had Hb Bart's levels ranging from 1.3 to 5.5% and a marked microcytosis and lowering of mean corpuscular haemoglobin (MCH) levels when compared with a control group of Black full-term neonates (table II).

Statistical analysis of the results showed a significant negative correlation between the Hb Bart's level and the mean cell haemoglobin of these babies (fig. 1). Postnatal follow-up of 4 of them showed that the abnormal component had disappeared after 4 months although the abnormality of the red cell indices persisted.

The parents of these 4 infants were examined and showed no haematological abnormalities indicative of thalassaemia. One mother had to be excluded because of iron deficiency (table III). Electrophoretic studies were normal and there was no evidence of HbH inclusions on redox dye-stained slides.

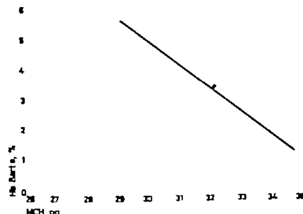


Fig. 1. Hb Bart's and MCH levels in South African Negro cord bloods.

possibly indicating a minor degree of imbalance of globin-chain synthesis during the switching-over process.

It is now well recognised that an increased blood level of Hb Bart's in the neonatal period is a sensitive indicator of α-thalassaemia in certain populations such as the Chinese, the Thais and Saudi Arabians. Other signs of α-thalassaemia, e.g. hydrops fetalis and HbH disease have also been reported in these groups.

The position is less clear in Negroid populations, with studies from America, Central and West Africa showing varying results although both qualitative and quantitative haemoglobinopathies are relatively common in these regions [11] The possible presence of an α-thalassaemia gene in the indigenous populations of the Southern African Continent has been suggested by a report of a single case of HbH disease in a Black adult and of Hb Bart's in two Zambian neonates [2]

The purpose of this study was to determine the significance of these isolated reports and to see whether there would be any correlation between the finding of Hb Bart's and the possible presence of α-thalassaemia. Such information would cast further light on the understanding of the thalassaemia mutations in indigenous Black peoples

The present survey was carried out in 1976 and 1977 and indicates the lowest reported incidence of elevated levels of Hb Bart's yet found in an indigenous Negroid population.

Materials and Methods

Unselected samples of placental cord blood were obtained from babies of mothers delivered in the Obstetrics Department of Baragwanath Hospital, Johannesburg. The blood was placed into ethylenediamine tetraacetic acid (EDTA) anticoagulant containers and red cell indices were determined using a Coulter Counter Model S [9]. Hb types were identified by cellulose-acetate electrophoresis in a glycine-EDTA Borate buffer pH 9.2 on a Gelman Microtek electrophoretic chamber [5] The Hb percentages were quantitated by elution spectrophotometry after electrophoresis in the same buffer using the semi-micro apparatus. The presence of a fast, anodal component was confirmed by starch-gel electrophoresis in a tris-EDTA-borate system, pH 8.5 [21].

Hb Bart's was further identified by cellulose-acetate electrophoresis in a 0.1 M phosphate buffer system, pH 7.0 and by globin electrophoresis in a 10 M urea-barbitone buffer system, pH 8.7 [17 19].

Hb H preparations were made after incubation of equal volumes of blood and 1% brilliant cresyl blue at 37 °C for 3 h.

Results

Of 430 cord bloods studied, 7 were found to have Hb Bart's (table I) No other abnormal Hb variants such as Hbs S C or Constant Spring were detected by this cellulose-acetate electrophoretic procedure. The

Table I. Haematological, electrophoretic and gestational data on 7 infants with Hb Bart's in their cord blood

Infant	Sex	Weight kg	Hb Bart's %	MCV fl	MCH pg
I.S.	F	2.80	5.5	88	28.2
C.M	F	3.10	[illegible].3	96	3[illegible].8
C.M	M	3.05	3.5	91	3[illegible].2
N.M	F	3.70	4.9	94	31.0
F.T	F	2.60	1.3	100	34.0
E.N	M	4.00	1.3	101	35.0
L.M	F	3.25	4.3	97	31.6

Table II. Haematological mean values of South African Negro neonates with Hb Bart's and age matched controls

	Controls (n = 77)		Infants with Hb Bart's (n = 7)	
	mean	SD	mean	SD
RBC, 10^6/µl	4.5	0.5	4.8	0.3
Hb, g/dl	15.6	1.5	15.4	1.9
PCV %	48.0	5.0	45.6	4.8
MCV fl	108.4	5.6	95.0	4.9
MCH, pg	37.2	1.8	32.1	2.3
MCHC, %	32.5	1.1	33.6	1.2

Highly significant difference between means of the two groups ($p < 0.001$).

Table III. Haematological mean values of the parents of Negro infants with Hb Bart's

	Controls (n = 20)		Parents (n = 7)	
	mean	SD	mean	SD
RBC, 10^6/µl	5.4	0.3	5.2	0.1
Hb, g/dl	14.2	1.3	13.9	1.5
PCV %	45.9	1.8	42.6	2.3
MCV fl	85	2.8	82	1.2
MCH, pg	36.3	0.4	36.7	0.6
MCHC, %	30.9	0.5	32.8	0.6

abnormal component was confirmed to be Hb Bart's on electrophoresis in the 0.1 *M* phosphate buffer system and also by the presence of only γ-chains on hypermolar urea globin electrophoresis.

The 7 infants with Hb Bart's showed no gestational evidence of prematurity as evidenced by their birth weights. They had Hb Bart's levels ranging from 1.3 to 5.5% and a marked microcytosis and lowering of mean corpuscular haemoglobin (MCH) levels when compared with a control group of Black full-term neonates (table II).

Statistical analysis of the results showed a significant negative correlation between the Hb Bart's level and the mean cell haemoglobin of these babies (fig. 1). Postnatal follow-up of 4 of them showed that the abnormal component had disappeared after 4 months although the abnormality of the red cell indices persisted.

The parents of these 4 infants were examined and showed no haematological abnormalities indicative of thalassaemia. One mother had to be excluded because of iron deficiency (*table III*). Electrophoretic studies were normal and there was no evidence of HbH inclusions on redox dye-stained slides.

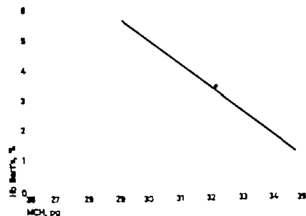


Fig. 1. Hb Bart's and MCH levels in South African Negro cord bloods.

possibly indicating a minor degree of imbalance of globin-chain synthesis during the switching-over process.

It is now well recognised that an increased blood level of Hb Bart's in the neonatal period is a sensitive indicator of α-thalassaemia in certain populations such as the Chinese, the Thais and Saudi Arabians. Other signs of α-thalassaemia, e.g. hydrops fetalis and HbH disease, have also been reported in these groups.

The position is less clear in Negroid populations, with studies from America, Central and West Africa showing varying results although both qualitative and quantitative haemoglobinopathies are relatively common in these regions [11]. The possible presence of an α-thalassaemia gene in the indigenous populations of the Southern African Continent has been suggested by a report of a single case of HbH disease in a Black adult and of Hb Bart's in two Zambian neonates [2].

The purpose of this study was to determine the significance of these isolated reports and to see whether there would be any correlation between the finding of Hb Bart's and the possible presence of α-thalassaemia. Such information would cast further light on the understanding of the thalassaemia mutations in indigenous Black peoples.

The present survey was carried out in 1976 and 1977 and indicates the lowest reported incidence of elevated levels of Hb Bart's yet found in an indigenous Negroid population.

Materials and Methods

Unselected samples of placental cord blood were obtained from babies of mothers delivered in the Obstetrics Department of Baragwanath Hospital, Johannesburg. The blood was placed into ethylenediamine tetraacetic acid (EDTA) anticoagulant containers and red cell indices were determined using a Coulter Counter Model S [9]. Hb types were identified by cellulose-acetate electrophoresis in a glycine EDTA Borate buffer pH 9.2 on a Gelman Microtek electrophoretic chamber [5]. The Hb percentages were quantitated by elution spectrophotometry after electrophoresis in the same buffer using the semi-micro apparatus. The presence of a fast, anodal component was confirmed by starch-gel electrophoresis in a tris-EDTA borate system, pH 8.5 [21].

Hb Bart's was further identified by cellulose-acetate electrophoresis in a 0.1 M phosphate buffer system, pH 7.0 and by globin electrophoresis in a 10 M urea-barbitone buffer system, pH 8.7 [17, 19].

Hb H preparations were made after incubation of equal volumes of blood and 1% brilliant cresyl blue at 37 °C for 3 h.

Results

Of 430 cord bloods studied, 7 were found to have Hb Bart's (table I). No other abnormal Hb variants such as Hbs S, C or Constant Spring were detected by this cellulose-acetate electrophoretic procedure. The

Table I. Haematological, electrophoretic and gestational data on 7 infants with Hb Bart's in their cord blood

Infant	Sex	Weight kg	Hb Bart's	MCV fl	MCH pg
I.S.	F	[illegible]	5.5	88	[illegible]
C.M	F	3 10	[illegible]	96	[illegible]
C.M	M	3.05	3.5	91	[illegible]
N.M	F	3 70	4 9	94	31.0
F T	F	[illegible]	1.3	100	34 0
E.N	M	4 00	1.3	101	35.0
L.M	F	[illegible]	4.3	97	31.6

Table II. Haematological mean values of South African Negro neonates with Hb Bart's and age-matched controls

	Controls (= 77)		Infants with Hb Bart's (n = 7)	
	mean	SD	mean	SD
RBC, $10^6/\mu l$	4.5	0.5	4.8	0.3
Hb, g/dl	15.6	1.5	15.4	1.9
PCV %	48.0	5.0	45.6	4.8
MCV fl	108.4	5.6	95.0	4.9
MCH, pg	37.2	1.8	32.1	2.3
MCHC, %	32.5	1.1	33.6	1.2

Highly significant difference between means of the two groups ($p < 0.001$).

Table III. Haematological mean values of the parents of Negro infants with Hb Bart's

	Controls (n = 20)		Parents (n = 7)	
	mean	SD	mean	SD
RBC, $10^6/\mu l$	5.4	0.3	5.2	0.1
Hb, g/dl	14.2	1.3	13.9	1.5
PCV %	45.9	1.8	42.6	2.3
MCV fl	85	2.8	82	1.2
MCH, pg	36.3	0.4	36.7	0.6
MCHC, %	30.9	0.5	32.8	0.6

abnormal component was confirmed to be Hb Bart's on electrophoresis in the 0.1 *M* phosphate buffer system and also by the presence of only γ-chains on hypermolar urea globin electrophoresis.

The 7 infants with Hb Bart's showed no gestational evidence of prematurity as evidenced by their birth weights. They had Hb Bart's levels ranging from 1.3 to 5.5% and a marked microcytosis and lowering of mean corpuscular haemoglobin (MCH) levels when compared with a control group of Black full-term neonates (table II).

Statistical analysis of the results showed a significant negative correlation between the Hb Bart's level and the mean cell haemoglobin of these babies (fig. 1). Postnatal follow-up of 4 of them showed that the abnormal component had disappeared after 4 months although the abnormality of the red cell indices persisted.

The parents of these 4 infants were examined and showed no haematological abnormalities indicative of thalassaemia. One mother had to be excluded because of iron deficiency (table III). Electrophoretic studies were normal and there was no evidence of HbH inclusions on redox dye-stained slides.

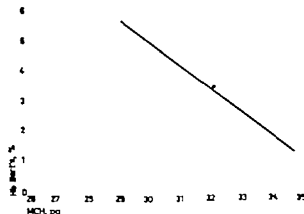


Fig. 1. Hb Bart's and MCH levels in South African Negro cord bloods.

Discussion

The finding that 1.6% of South African Negro newborns may carry an α-thalassaemia gene raises certain questions about the genetic affinities of this population with other Negroid groups. From table IV it will be seen that there is a great variation in the proportion of neonates with elevated Hb Bart's levels among Negroes both in Africa and in the New World. The exceptionally high incidence found by *Horton et al.* [7] among US Blacks may simply be the result of very sensitive staining techniques used by them, detecting levels of Hb Bart's lower than 0.5%. The low incidence reported here may be indicative of a valid genetic distinction between sub-Zambezian Negroid peoples and those of the equatorial region.

The present study has shown that 1.6% of 430 South African Negro neonates have Hb Bart's levels greater than 1 and less than 6%. There is haematological evidence of a microcytosis and a low MCH in these infants, but not in their parents. Comprehensive investigation of the parents failed to reveal any haemoglobinopathy. The early disappearance of the Hb Bart's in the babies with persistence of the microcytosis and low MCH is apparent.

Clinical experience has shown a virtual absence of overt quantitative α-chain disorders in this local population, i.e. Hb Bart's hydrops fetalis and HbH disease have never been reported [11, 23]. However objective evidence of a persistent microcytosis and low MCH in the infants with neonatal Hb Bart's has been shown in this survey. In the absence of anaemia this finding is highly suggestive of α-thalassaemia [17, 22]. There is also a strong negative correlation between the quantity of Hb Bart's and the MCH. All these findings point to the presence of a type of α-thalassaemia rather than a transient developmental abnormality as suggested by *Folayan Esan* [4] and *Schmaier et al* [16].

Genetic and clinical evidence indicates that there is more than one type of α-thalassaemia [20]. Significantly Hb Bart's hydrops fetalis has never been described in Blacks so that the existence of a different α-thalassaemia gene in African Negroes from that found in the Mediterranean, Middle Eastern and Oriental populations can already be deduced [10, 14, 18, 21]. Furthermore, evidence for heterogeneity of α-thalassaemia among indigenous African populations is suggested by the present study. The infrequent reports of α-thalassaemia syn-

Table IV. The incidence of Hb Bart's in cord blood in various Negro groups

Ethnic group	Number studied	Incidence,	Authors, year and reference
U.S. Negro	449	7.1	*Minnich et al.*, 196_ [12]
U.S. Negro	300	30.0	*Horton et al* 1962 [7]
Senegalese	345	1.75	*Oudart et al* 1968 [13]
Congolese	636	17.9	*Van Baelen et al* 1969 [20]
Nigerian	140	10.7	*Hendrickse et al* 1960 [6]
Nigerian	1,866	5.1	*Folayan Esan*, 1972 [4]
S.A. Negro	430	1.6	present study

dromes among the indigenous peoples of Central and Southern Africa may reflect a lack of sophisticated diagnostic techniques, such as globin chain synthesis, throughout the Continent. Such techniques would be necessary for the recognition of a milder African α-thalassaemia gene, the expression of which in the sub-Zambezian Black is characterised by minimal reduction of α-chain synthesis. The finding of a lower incidence and lower levels of Hb Bart's in cord blood samples is further proof of this hypothesis [15]. Further light may be expected to be shed on this problem by Hb biosynthetic studies on selected infants and their parents.

Acknowledgements

The author thanks Prof. S. *Wayburne* and Prof. L. *van Dongen*, and the nursing staff at Baragwanath Hospital, for their help in the collection of blood samples; Prof. *Jack Katz*, Head of the Department of Haematology, The South African Institute for Medical Research, for facilities, and Prof. T. *Jenkins* for his interest and encouragement.

References

1 Ager, J. A. M. and Lehmann, H.: Observations on some 'fast' haemoglobins: K, J, N and 'Bart's'. Br. med. J. *i*: 929–930 (1958).

2 Barclay, G. P. T., Charlesworth, D., and Lehmann, H.: Abnormal haemoglobins in Zambia. A new haemoglobin Zambia α60 (E9) lysine → asparagine. Br. med. J. *iv*: 595–596 (1969).

3 Fessas, P. H. and Papaspyrou, A.: New 'fast' hemoglobin associated with thalassemia. Science, N.Y. *126*: 1119–1120 (1957).

4 Folayan Esan, G. J.: Haemoglobin Bart' in newborn Nigerians. Br. J. Haemat. *22*: 73–80 (1972).

5 Gelman Technical Bulletin 22 (Gelman Instrument Co., Ann Arbor 1975).

6 Hendrickse, R. G., Boyd, A. E., Fitzgerald, P. A., and Ransome Kuti, S.: Studies on the haemoglobins of newborn Nigerians. Br. med. J. *i*: 611–613 (1960).

7 Horton, B. F., Thompson, R. B., Dozy, A. M., Nechtman, C. M., Nichols, E., and Huisman, T. H. J.: In Homogeneity of hemoglobin. VI. The minor hemoglobin components of cord blood. Blood *20*: 302–310 (1962).

8 Hunt, J. A. and Lehmann, H.: Haemoglobin Bart's: a foetal haemoglobin without α-chains. Nature, Lond. *184*: 872–874 (1959).

9 Instructions Manual for the Model's Coulter Counter (Coulter Electronics, Hialeah 1969).

10 Lehmann, H.: Different types of alpha thalassaemia and significance of haemoglobin Bart's in neonates. Lancet *ii*: 27–28 (1970).

11 Livingstone, F. B.: Abnormal haemoglobins in human populations (Aldine, Chicago 1967/1972).

12 Minnich, V., Cordonnier, J. J., Williams, W. J., and Moore, C. V.: Alpha, beta and gamma globulin polypeptide chains during the neonatal period with description of fetal form of hemoglobin D αSt. Louis. Blood *19*: 137–140 (1962).

13 Oudart, J. L., Diadhiou, F., Sarrat, H. et Satge, P.: L'hémoglobine du nouveau-né Africain. Annls paediat. *50*: 3073–3081 (1968).

14 Pembrey, M. E., Weatherall, D. J., Clegg, J. B., Bunch, C., and Perrine, R. P.: Haemoglobin Bart's in Saudi Arabia. Br. J. Haemat. *29*: 221–230 (1975).

15 Pootrakul, S. and Wasi, P. *et al.*: Studies on Hb Bart's in Thailand: the incidence and the mechanism of occurrence in cord blood. Ann. hum. Genet. *31*: 149–153 (1967).

16 Schmaier, A. H., *et al.*: Alpha thalassemia screening in neonates by mean corpuscular volume and mean corpuscular hemoglobin determination. *J. Pediat. 83*: *794–797 (1973).*

17 Schneider, R. G.: Developments in laboratory diagnosis; in Abramson, Bertles and Wethers: Sickle cell disease diagnosis, management, education and research (Mosby, St. Louis 1973).

18 Schwartz, E. and Atwater, J.: α-Thalassaemia in the American Negro. J. clin. Invest. *51*: 412–415 (1972).

*19 Ueda, S. and Schneider, R. G.: Rapid differen*tiation of polypeptide chains of hemoglobin by

cellulose acetate electrophoresis of hemolysates. Blood *34* 230–232 (1969)

20 Van Baelen, H. Van De Pitte, J Cornu, G and Beckles, R. Routine detection of sickle cell anaemia and haemoglobin Bart's in Congolese neonates. Trop. geogr Med. *21* 412–415 (1969).

21 Weatherall D J and Clegg, J B.. The thalassaemia syndromes 2nd ed. (Blackwell Scientific Publications, Oxford 1972).

22 Weatherall, D J.. Abnormal haemoglobins in the neonatal period and their relationship to thalassaemia. Br J Haemat. 9 265–275 (1963).

23 Pillsrzek, T S.. Unpublished personal observations.

Received. October 4 1977
Accepted. July 11 1978

Dr T S. Pillsrzek, Department of Haematology
The South African Institute for
Medical Research, and School of Pathology
University of the Witwatersrand, Johannesburg
(South Africa).

Acta haemat. 61: 39–46 (1979)

Occurrence of Haemoglobin Norfolk ($\alpha_2{}^{57(E6)\ Gly \to Asp}\beta_2$) at the Level of 33% in an Italian Family from Calabria

M. Marinucci, F. Mavilio, P. Samoggia, L. Tentori, G. Spadea and G. Cocone

Laboratorio di Patologia non Infettiva, Istituto Superiore di Sanità, Rome, and Ospedale Civile A. Pugliese, Catanzaro

Key Words. Abnormal haemoglobins · Hb Norfolk · α-Chain loci · Genetic polymorphism

Abstract. An abnormal, fast-moving haemoglobin was observed in 5 healthy subjects of a family from Calabria (southern Italy). In all these carriers the abnormal haemoglobin, which structural studies identified as Hb Norfolk ($\alpha_2{}^{57(E6)\ Gly \to Asp}\beta_2$) [4], occurs at a level averaging 33% of the total haemoglobin. Biosynthetic studies showed no evidence for unbalance of the globin chain synthetic ratio. In order to account for the observed percentages of Hb Norfolk, current concepts about the α-globin chain genetic system are reviewed, and different genic arrangements which would be in agreement with the experimental findings are discussed.

Introduction

The question of whether there exists a polymorphism for the number of the α-globin chain structural loci and/or for their relative expression among and within certain human populations is now of considerable interest. A complete knowledge of whether and how different numbers of α-chain loci per diploid complement influence the globin chain production could be of great importance in explaining the genetic mechanisms underlying the various types of α-thalassaemia syndromes in man.

As all the normal α-gene products are structurally undistinguishable, a common approach to the study of the number of α-genes and of their genetic expression is the observation of the modalities of inheritance and of the relative proportions of one or more α-chain variants in families in which they segregate. These studies have so far demonstrated the presence of multiple structural loci governing the α-chain production in man and, moreover, they seem to provide evidence for the existence of a genetic polymorphism of the α-chain haplotypes (for a review see *Rucknagel and Winter* [25]). Nevertheless, the entire question of the α-genes system is far from being completely elucidated, and a more complete collection of data concerning families with

α-chain structural haemoglobin variants will be helpful for a better understanding

Haemoglobin Norfolk ($\alpha_2{}^{57\,(E6)\,Gly\rightarrow Asp}\,\beta_2$) is a relatively rare variant. It was first described as a fast moving haemoglobin in a young Englishman in Singapore by *Ager et al* [2] while the amino acid substitution was later identified by *Baglioni* [3] Hb Norfolk has been successively reported in association with β-thalassaemia, in an Italian family of Calabrian extraction living in Australia [33] and in Japanese subjects [26] More recently Hb Norfolk was noted in 3 unrelated Indian soldiers, i e. Nepali Gurkhas [17] and, in association with β-thalassaemia, in another Italian family from Calabria [19] The occurrence of this abnormal haemoglobin in such different ethnic groups strongly suggests that it might have arisen by independent mutations.

In both the Italian families studied to date the level of Hb Norfolk in the heterozygotes ranges from 35 to 37% [33] and from 33 to 45% [19] These amounts are significantly higher than both the 27% observed in the English carrier [2] and the 25% reported in Japanese carriers [26] (unfortunately the level of Hb Norfolk in Nepali Gurkhas has *not so far been indicated*) These findings would suggest that the same mutation has occurred in genotypes formed by either different number of α-chain loci or by loci having different expressions.

The presence of Hb Norfolk does not appear to produce appreciable clinical disorders, and the abnormal haematological picture shown by thalassaemic carriers has in all likelihood to be ascribed to the sole β-thalassaemia trait. *It is noteworthy that in* these carriers the level of Hb Norfolk does not appear to be decreased in comparison with the normal carriers, contrary to other known cases of interaction between β-thalassaemia determinant and mutant structural α-chain genes (see *Marinucci et al* [16])

This paper describes the occurrence of Hb Norfolk at a level of about 33% in 5 subjects from an Italian family living in Crotone (Calabria). Three alternative genetic models which would account for the observed amount of Hb variant are discussed.

Materials and Methods

Venous blood was collected in heparinized tubes, and standard haematological methods, including Coulter counter model S analysis, were employed [11]. Isopropanol [9] and heat [31] stability tests were carried out on whole haemolysates. Electrophoretic analyses of the haemolysates were carried out on cellulose-acetate plates (Titan III Helena Laboratories, Beaumont, Tex.) in a Tris-EDTA-borate buffer pH 8.4 and haemoglobin fractions were quantitated by an elution method [15] as the mean of ten determinations. The technique was highly reproducible (coefficient of variation < 4%) The level of Hb F was estimated by alkali denaturation [7]. The isoelectric points of the haemoglobin fractions were measured by thin-layer isoelectric focusing (IEF) on polyacrylamide plates [8] using a LKB Multiphor system (LKB Productor Bromma, Sweden). Globin chains were separated by CM-cellulose column chromatography [10], digested with trypsin (TPCK, Worthington Biochem.) [27] and finger printed [3]. Peptides were directly eluted from preparative fingerprints with 5.7 N HCl and hydrolysed in sealed evacuated tubes for 18 h at 105 °C. Amino acid analyses were carried out on Beckman 120 B equipment. Dansyl-Edman degradation [28] was carried out on peptides eluted from paper with 0.01 N NH OH. *In vitro* biosynthesis of haemoglobin was performed by incubating for 60 min reticulocyte-enriched red cells from peripheral blood [32] using [^{3}H]-leucine as marker After lysis and removal of stroma by centrifugation, globin was prepared and chromatographed as described above, and radioactivity of each fraction was assayed by liquid scintillation using a Beckman LS-150 system.

Results

A pedigree of the family is shown in figure 1 and haematological and haemoglobin data from its members are summarized in table I. All of them showed normal clinical and haematological pictures, with normal red blood cell indices and morphology and no evidence of haemolysis *in vivo* or *in vitro* On supravital staining no inclusion bodies were observed in the red cells. A slight decrease of the red cell haemoglobin content and/or of the red cell count in some young subjects is most probably due to inadequate diet.

In five members of the family (I 1 II-4 II-6, II 7 and II-9) cellulose-acetate electrophoresis of the haemolysate showed an abnormal haemoglobin band with the same mobility of Hb J amounting to about 33% of the total haemoglobin. A minor component, more anodic than Hb A, was also detected, most likely corresponding to Hb J_2 ($\alpha^J_2\delta_2$). The isoelectric point of the abnormal haemoglobin was 6.79 + 0 02 (mean + SE of eight determinations). All of the tests used to look for evidence of haemoglobin instability yielded negative results.

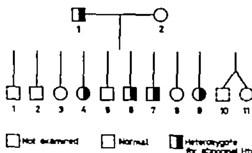


Fig. 1. Family pedigree.

On the fingerprint obtained from isolated abnormal α-chains the peptides αTp VII (residues 57–60) and α-Tp VII–VIII (residues 57–61) were replaced by two more negatively charged ones in both of them the amino acid analysis showed an additional residue of aspartic acid and a missing glycine. In order to determine the position in

Table I. Haematological and haemoglobin data

Subject	Age years	RBC 10^{12}/l	Hb g/dl	PCV	MCV fl	MCH pg	MCHC g/dl	Hb Norfolk %	Hb A_2 %	Hb J_2 %	Hb F %	Biosynthetic ratios J/β	Biosynthetic ratios α^J/α	Absolute amounts, pg/cell α^A chain	Absolute amounts, pg/cell α^J chain
I-1		4.64	13.9	0.38	82	30.2	36 8	33.2	1 4	0.4	1.1	1.02	0.38	10.1	5 0
I-2		4.56	14.2	0.41	91	31.3	34.3		2.5	–	0.5	1.05	–	15.65	
II 2	13	4 76	14.2	0.42	89	30 0	33.0	–	3.0	–	0.5	0.95	–	15.0	–
II-3	14	4.05	12.3	0.38	85	27 4	32.5	–	2.4	–	1 4	1.04	–	13.7	
II-4	9	4.45	12.1	0.37	82	27 4	33 4	32.6	1.8	0.5	1.6	1.08	0.35	9.25	4.45
II 5	8	4.39	11.9	0.36	82	27.2	33.2		2.0	–	1.5	0.99	–	13.6	–
II-6	6	4.31	12.4	0.37	87	28.9	33.5	33.1	1.6	0.5	1.4	1.06	0.37	9.65	4 8
II-7	5	4.25	12.8	0.39	92	30.3	32.9	32.7	1.9	0.6	1.2	1.10	0.35	10.2	4.95
II-8	4	4.56	12.4	0.37	82	27 4	33.7		2.3	–	1 7	0.91	–	13.7	
II-9	1.5	4 68	14.9	0.43	92	32.0	33.7	33.4	1.3	0.4	1.9	0.96	0.36	10.65	5.35

α-chain structure, heterozygous variants will be needed for a better understanding.

Haemoglobin Norfolk [α57(E6) Gly→Asp] is a relatively rare variant. It was first described as a fast-moving haemoglobin in a young Englishman in Singapore by Ager et al. [illegible], while the amino acid substitution was later identified by Baglioni [illegible]. Hb Norfolk has been subsequently reported in association with β-thalassaemia in an Indian family of Caucasian extraction living in Australia [illegible], and in Japanese subjects [illegible]. More recently, Hb Norfolk was noted in 3 unrelated Indian subjects in Newark, Gardiner [illegible], and in association with β-thalassaemia in another Indian family from Calabria [illegible]. The occurrence of this abnormal haemoglobin in such different ethnic groups strongly suggests that it might have arisen by independent mutations.

In both the Indian families studied to date the level of Hb Norfolk in the heterozygous ranges from [illegible] to [illegible]% [illegible] and from [illegible] to [illegible]% [illegible]. These amounts are significantly lower than both the [illegible]% observed in the English carrier [illegible] and the [illegible]% reported in Japanese carriers [illegible]. Unfortunately the level of Hb Norfolk in Newark Gardiner has not so far been published. These findings would suggest that the same mutation has occurred in genomes formed by either different number of α-globin loci or by loci having different expression.

The presence of Hb Norfolk does not appear to produce appreciable clinical disorders, and the abnormal haematological picture shown by thalassaemic carriers has in all likelihood to be ascribed to the sole β-thalassaemia trait. It is noteworthy that in these carriers the level of Hb Norfolk does not appear to be decreased in comparison with the normal carriers, contrary to other known cases of interaction between β-thalassaemia determinants and mutant structural α-chain genes (see Marinucci et al. [illegible]).

This paper describes the occurrence of Hb Norfolk at a level of about [illegible]% in 3 subjects from an Italian family living in Cosenza (Calabria). Three alternative genetic models which would account for the observed amount of Hb variant are discussed.

Materials and Methods

Venous blood was collected in heparinized tubes, and standard haematological methods, including Coulter counter model S analysis, were employed [illegible]. Isopropanol [illegible] and heat [illegible] stability tests were carried out on whole haemolysates. Electrophoretic analyses of the haemolysates were carried out on cellulose-acetate plates (Titan III, Helena Laboratories, Beaumont, Tex.) in a Tris-EDTA-borate buffer pH 8.6, and haemoglobin fractions were quantitated by an elution method [illegible] as the mean of two determinations. The technique was highly reproducible (coefficient of variation <4%). The level of Hb F was estimated by alkali denaturation [illegible]. The isoelectric points of the haemoglobin fractions were measured by thin-layer isoelectric focusing [illegible] on polyacrylamide plates [illegible] using a LKB Multiphor system (LKB Produkter, Bromma, Sweden). Globin chains were separated by CM-cellulose column chromatography [illegible], digested with trypsin (TPCK, Worthington Biochem.) [illegible] and fingerprinted [illegible]. Peptides were directly eluted from preparative fingerprints with [illegible] HCl and hydrolysed in sealed evacuated tubes for [illegible] h at 110 °C. Amino acid analyses were carried out on Beckman [illegible] equipment. Dansyl-Edman degradation [illegible] was carried out on peptides eluted from paper with [illegible]. In vitro biosynthesis of haemoglobin was performed by incubating for [illegible] min reticulocyte-enriched red cells from peripheral blood [illegible] using [illegible]-leucine as marker. After lysis and removal of stroma by centrifugation, globin was prepared and chromatographed as described above, and radioactivity of each fraction was assayed by liquid [illegible] using a Beckman LS-[illegible] system.

Results

A pedigree of the family is shown in figure 1 and haematological and haemoglobin data from its members are summarized in table I. All of them showed normal clinical and haematological pictures, with normal red blood cell indices and morphology and no evidence of haemolysis *in vivo* or *in vitro*. On supravital staining no inclusion bodies were observed in the red cells. A slight decrease of the red cell haemoglobin content and/or of the red cell count in some young subjects is most probably due to inadequate diet.

In five members of the family (I 1 II-4 II-6 II 7 and II-9), cellulose acetate electrophoresis of the haemolysate showed an abnormal haemoglobin band with the same mobility of Hb J amounting to about 33% of the total haemoglobin. A minor component, more anodic than Hb A, was also detected, most likely corresponding to Hb J_2 ($\alpha^J_2\delta_2$). The isoelectric point of the abnormal haemoglobin was 6.79 + 0 02 (mean + SE of eight determinations). All of the tests used to look for evidence of haemoglobin instability yielded negative results.

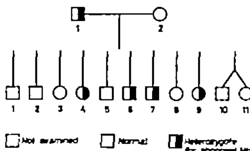


Fig. 1. Family pedigree.

On the fingerprint obtained from isolated abnormal α-chains the peptides αTp VII (residues 57–60) and α-Tp VII–VIII (residues 57–61) were replaced by two more negatively charged ones, in both of them the amino acid analysis showed an additional residue of aspartic acid and a missing glycine. In order to determine the position in

Table I. Haematological and haemoglobin data

Subject	Age years	RBC $10^{12}/l$	Hb g/dl	PCV	MCV fl	MCH pg	MCHC g/dl	Hb Norfolk %	Hb A_2 %	Hb J_2 %	Hb F %	Biosynthetic ratios		Absolute amounts, pg/cell	
												α/β	α^J/α	α^A-chain	α^J-chain
I-1		4 64	13.9	0.38	82	30.2	36 8	33.2	1 4	0.4	1.1	1.02	0.38	10.1	5.0
I 2		4.56	14.2	0.41	91	31.3	34.3		2.5	–	0.5	1.05	–	15.65	
II-2	13	4.76	14.2	0.42	89	30 0	33.0		3.0	–	0.5	0.95		15.0	–
II-3	14	4.05	12.3	0.38	85	27 4	32.5		2.4		1.4	1.04		13.7	
II-4	9	4.45	12.1	0.37	82	27 4	33.4	32.6	1.8	0.5	1.6	1.08	0.35	9.25	4.45
II 5	8	4.39	11.9	0 36	82	27.2	33.2	–	2.0	–	1.5	0.99	–	13.6	–
II-6	6	4.31	12.4	0.37	87	28.9	33.5	33.1	1 6	0.5	1 4	1.06	0.37	9.65	4.8
II-7	5	4.25	[illegible]	0.39	92	30.3	32.9	32.7	1.9	0.6	1.2	1.10	0.35	10.2	4.95
II-8	4	4.56	12.4	0.37	82	77 4	33.7		2.3	–	1 7	0.91	–	13.7	–
II-9	1.5	4.68	14.9	0.43	97	32.0	33.7	33.4	1.3	0.4	1.9	0.96	0.36	10.65	5.35

α-chain structural haemoglobin variants will be helpful for a better understanding.

Haemoglobin Norfolk ($\alpha_2^{57\,(E6)\,Gly\rightarrow Asp}\beta_2$) is a relatively rare variant. It was first described as a fast moving haemoglobin in a young Englishman in Singapore by *Ager et al* [2] while the amino acid substitution was later identified by *Baglioni* [3]. Hb Norfolk has been successively reported, in association with β-thalassaemia, in an Italian family of Calabrian extraction living in Australia [33] and in Japanese subjects [26]. More recently Hb Norfolk was noted in 3 unrelated Indian soldiers, i.e. Nepali Gurkhas [17] and in association with β thalassaemia, in another Italian family from Calabria [19]. The occurrence of this abnormal haemoglobin in such different ethnic groups strongly suggests that it might have arisen by independent mutations.

In both the Italian families studied to date the level of Hb Norfolk in the heterozygotes ranges from 35 to 37% [33] and from 33 to 45% [19]. These amounts are significantly higher than both the 27% observed in the English carrier [2] and the 25% reported in Japanese carriers [26] (unfortunately the level of Hb Norfolk in Nepali Gurkhas has not so far been indicated). These findings would suggest that the same mutation has occurred in genotypes formed by either different number of α-chain loci or by loci having different expressions.

The presence of Hb Norfolk does not appear to produce appreciable clinical disorders, and the abnormal haematological picture shown by thalassaemic carriers has in all likelihood to be ascribed to the sole β-thalassaemia trait. It is noteworthy that in these carriers the level of Hb Norfolk does not appear to be decreased in comparison with the normal carriers, contrary to other known cases of interaction between β-thalassaemia determinant and mutant structural α-chain genes (see *Marinucci et al* [16]).

This paper describes the occurrence of Hb Norfolk at a level of about 33% in 5 subjects from an Italian family living in Crotone (Calabria). Three alternative genetic models which would account for the observed amount of Hb variant are discussed.

Materials and Methods

Venous blood was collected in heparinized tubes, and standard haematological methods, including Coulter counter model S analysis, were employed [11]. Isopropanol [9] and heat [31] stability tests were carried out on whole haemolysates. Electrophoretic analyses of the haemolysates were carried out on cellulose-acetate plates (Titan III, Helena Laboratories, Beaumont, Tex.) in a Tris-EDTA-borate buffer pH 8.4 and haemoglobin fractions were quantitated by an elution method [15] as the mean of ten determinations. The technique was highly reproducible (coefficient of variation < 4). The level of Hb F was estimated by alkali denaturation [7]. The isoelectric points of the haemoglobin fractions were measured by thin-layer isoelectric focusing (IEF) on polyacrylamide plates [8] using a LKB Multiphor system (LKB Productor, Bromma, Sweden). Globin chains were separated by CM-cellulose column chromatography [10], digested with trypsin (TPCK, Worthington Biochem.) [27] and fingerprinted [3]. Peptides were directly eluted from preparative fingerprints with 5.7 *N* HCl and hydrolysed in sealed evacuated tubes for 18 h at 105 °C. Amino acid analyses were carried out on Beckman 120 B equipment. Dansyl-Edman degradation [28] was carried out on peptides from paper with 0.01 *N* NH OH. *In vitro* thesis of haemoglobin was performed by ing for 60 min reticulocyte-enriched red cells peripheral blood [32] using [³H]-leucine as marker. After lysis and removal of stroma by trifugation, globin was prepared and graphed as described above, and radioactivity each fraction was assayed by liquid using a Beckman LS-150 system.

Results

A pedigree of the family is shown in figure 1 and haematological and haemoglobin data from its members are summarized in table I. All of them showed normal clinical and haematological pictures, with normal red blood cell indices and morphology and no evidence of haemolysis *in vivo* or *in vitro* On supravital staining no inclusion bodies were observed in the red cells. A slight decrease of the red cell haemoglobin content and/or of the red cell count in some young subjects is most probably due to inadequate diet.

In five members of the family (I 1, II-4 II-6 II 7 and II 9), cellulose-acetate electrophoresis of the haemolysate showed an abnormal haemoglobin band with the same mobility of Hb J amounting to about 33 /₀ of the total haemoglobin. A minor component, more anodic than Hb A, was also detected, most likely corresponding to Hb J ($\alpha^J_2\delta_2$) The isoelectric point of the abnormal haemoglobin was 6.79 + 0 02 (mean + SE of eight determinations) All of the tests used to look for evidence of haemoglobin instability yielded negative results.

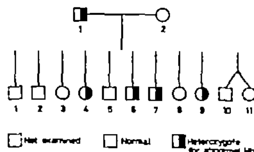


Fig. 1. Family pedigree.

On the fingerprint obtained from isolated abnormal α-chains the peptides αTp VII (residues 57–60) and α-Tp VII–VIII (residues 57–61) were replaced by two more negatively charged ones In both of them the amino acid analysis showed an additional residue of aspartic acid and a missing glycine. In order to determine the position in

Table I. Haematological and haemoglobin data

Subject	Age years	RBC 10^{12}/l	Hb g/dl	PCV	MCV fl	MCH pg	MCHC g/dl	Hb Norfolk %	Hb A_2 %	Hb J_2 %	Hb F %	Biosynthetic ratios γ/β	Biosynthetic ratios $\alpha^J/$	Absolute amounts, pg/cell α^A-chain	Absolute amounts, pg/cell α^J chain
I-1		4.64	13.9	0.38	82	30.2	36.8	33.2	1 4	0.4	1.1	1.02	0.38	10.1	5 0
I-2		4.56	14.2	0.41	91	31.3	34.3	–	2.5	–	0.5	1.05	–	15.65	
II-2	13	4.76	14.2	0.42	89	30.0	33.0	–	3 0		0.5	0.95		15.0	–
II-3	14	4 05	12.3	0.38	83	27 4	32.5		2.4		1 4	1.04		13.7	
II-4	9	4 45	12.1	0.37	82	27 4	33.4	32.6	1.8	0.5	1.6	1.08	0.35	9.23	4.45
II-5	8	4.39	11.9	0.36	82	27.2	33.2	–	2.0		1.5	0.99		13.6	
II-6	6	4 31	12.4	0.37	87	28.9	33.5	33.1	1.6	0.5	1 4	1.06	0.37	9.63	4.8
II 7	5	4.25	[illegible]	0.39	92	30.3	32.9	32.7	1.9	0 6	1.2	1.10	0.35	10.2	4.95
II-8	4	4.36	12.4	0.37	82	77 4	33.7	–	2.3		1.7	0.91	–	13.7	–
II-9	1.5	4.68	14.9	0.43	9?	32.0	33.7	31.4	1.3	0.4	1.9	0.96	0.36	10 63	5.35

which a glycine had been replaced by an aspartic acid (α^{57} or α^{59}) the abnormal peptides were submitted to sequential degradation and in both cases the identification of the N terminal residue by the dansyl-chloride technique proved the amino acid substitution to be $\alpha^{57\ Gly \rightarrow Asp}$ as in Hb Norfolk [4]

Biosynthetic results are reported in table I all subjects were found to have an overall α/non-α-chain synthesis ratio within the normal range, and moreover the $\alpha^{Norfolk}$/total α-ratios from the five Hb Norfolk carriers appeared in good agreement with the respective proportions of Hb variant

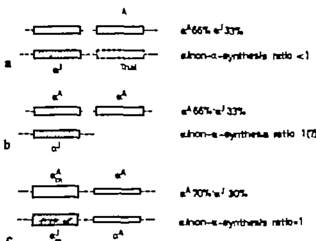


Fig. 2. Three different genic arrangements which account for a level of about 33% of abnormal haemoglobin. For simplicity α-genes are assumed to be linked. Variant genes are shaded. α_m and α_l represent *more* and *less* producing genes (for explanation see text).

Discussion

Structural Studies

In the steric model proposed by *Perutz* [21] for Hb A residue 57 occupies the sixth position in the helical E segment of the α-chain next to the distal haem-linked histidine residue α^{58} (E7) Nevertheless, the substitution from Gly to Asp occurring at this position in the Hb Norfolk molecule does not appear to influence its stability or its functional properties, at least as far as clinical symptoms are concerned. In addition, it shows no abnormalities in the haem-binding as in the tendency to methaemoglobin formation [24] It is worth noting that the only other known haemoglobin with a substitution of the same residue, Hb L Persian Gulf ($\alpha^{57\ Gly \rightarrow Arg}$) [24] similarly produces no appreciable clinical consequences and that position E6 shows indeed a wide phylogenetic variability in other known globins [12]

Genetic Considerations

In all Hb Norfolk heterozygous carriers of this family the father and 4 siblings, the abnormal haemoglobin occurs at a constant level averaging 33%, which can be accounted for on the basis of at least three different hypotheses concerning possible underlying genic arrangements.

(a) According to the 4 α-chain genes model proposed by *Lehmann and Carrell* [14] the observed proportion of abnormal haemoglobin can be explained assuming the presence of two active α^A loci on one chromosome and one α^J locus on the other one, the second locus on this chromosome having been involved in an α-thalassaemia mutation (i.e., the $\alpha^A\alpha^A/\alpha^J\alpha^{thal}$ arrangement, see figure 2a) This hypothesis has been first postulated in order to account for the 33% level of Hb G Philadelphia (α^{68} Asn→Lys) observed in a number of carriers by *French and Lehmann* [13] and later by *Politis T gos et al* [22] This arrangement is to cause a defective α-chain which would result in the slight of the α/non-α-chain synthesis ratio

of the silent form of α-thalassaemia (α-thal₂ trait) as already observed by the above-mentioned authors. In the present case, on the grounds of our biosynthetic results, this model can be reasonably ruled out.

(b) The second hypothesis is based upon the assumption that there exists a polymorphism for the number of α-chain structural loci in man. There is now much evidence that in certain human populations, individuals may have one active α-chain locus per chromosome, while in other populations two active loci are present [25]. Variable gene dosage *within* the same population has been first postulated in order to account for the well-defined trimodal distribution of percent levels of Hb G $\alpha^{Philadelphia}$ observed in American Black populations by *Milner and Huisman* [18] and *Baine et al.* [5]. *Trabuchet et al.* [29] also proposed variable gene dosage as the cause of the bimodal distribution (31 and 40%) observed for Hb J Mexiko ($\alpha^{54\ Gln \to Glu}$) in the Algerian population. According to these authors, the proportion of α-chain variants around means of approximately 20–25, 30–33 and 40–45% can be explained by assuming four, three or two active loci respectively per diploid complement, one of them bearing the amino acid substitution (i.e., $\alpha^X\alpha^A/\alpha^A\alpha^A$, $\alpha^X/\alpha^A\alpha^A$ or $\alpha^X\alpha^A/\alpha^A$, α^X/α^A). *Milner and Huisman* [18] observed in a study of eight families that *percentages of Hb G around means of 33%* were associated with slightly unbalanced synthetic ratio, while percentages around means of 46% were constantly associated with microcytosis, hypochromia and considerably impaired synthetic balance. Nevertheless, the authors excluded the possibility of a linked α-thalassaemia. These findings would provide evidence that the human α-genes always possess approximately the same quantitative expression, and hence genotypes containing less than four active loci give rise to proportional α-chain deficit, and consequently to haematological pictures resembling the mild forms of α-thalassaemia. The authors also suggest that the α-thalassaemia forms of black Africans could be simply due to both $\alpha^A/{}^A$ and $\alpha^A/\alpha^A\alpha^A$ genotypes. However it remains unclear whether these genotypes necessarily cause a total α-chain deficit. *Baine et al.* [5] in fact, in their study of 53 black American heterozygotes for Hb G $\alpha^{Philadelphia}$ observed the percentage of Hb variant to be negatively correlated with the mean cell volume (MCV) and the mean cell haemoglobin (MCH) content, but no evidence for unbalanced chain synthesis was found in both 30 and 40% Hb G classes. The authors suggest that transcriptional and/or translational control mechanisms limiting the complementary chain production could balance the decreased absolute amount of α-chain synthetized by the lowered α-gene dosage (the α-thalassaemia abnormalities would be due both to diminished α-chain synthesis and absence of the balancing control mechanism(s)).

Moreover, heterozygous and homozygous subjects for Hb J Tongariki ($\alpha^{115\ Ala \to Asp}$) (proposed genotypes α^J/α^A and α^J/α^J respectively [1, 6]) and heterozygotes with 40–45% of Hb Anantharaj ($\alpha^{11\ Lys \to Glu}$) [23] seem to show no haematological or clinical evidence of α-thalassaemia.

All these observations allow to postulate the existence of at least two different haplotypes containing a single active α-chain locus, one which causes unbalanced globin chain synthesis, and the other which seems not to. Then, it is tempting to extrapolate, assuming the α-gene system to be at present

[illegible]

which a globin has been [illegible] (? or ?) the abnormal [illegible] [illegible] and in both cases the [illegible] of [illegible] [illegible] [illegible] [illegible]

[illegible] results [illegible] in table [illegible]

Discussion

[illegible]

[illegible]

Fig. 2. Three different gene arrangements which account for a loss of about [illegible] of [illegible] haemoglobin. [illegible]

abnormal haemoglobin occurs at a constant level, averaging [illegible], which can be accounted for on the basis of at least three different hypotheses concerning possible underlying gene arrangements.

According to the [illegible] model proposed by Lehmann and Carrell [illegible] the observed proportion of abnormal haemoglobin can be explained assuming the presence of two pairs of loci on one chromosome and one [illegible] locus on the other [illegible] the second locus on this chromosome having been involved in an [illegible] [illegible] This hypothesis has been first [illegible] in order to account for the [illegible] level [illegible] observed in a number of [illegible] by [illegible] and Lehmann [illegible] and later by [illegible] [illegible] This arrangement is [illegible] to cause [illegible] which would result in the slight imbalance of the [illegible] synthesis [illegible]

[illegible]

of the silent form of α-thalassaemia (α-thal$_2$ trait), as already observed by the above-mentioned authors. In the present case, on the grounds of our biosynthetic results, this model can be reasonably ruled out.

(b) The second hypothesis is based upon the assumption that there exists a polymorphism for the number of α-chain structural loci in man. There is now much evidence that in certain human populations, individuals may have one active α-chain locus per chromosome, while in other populations two active loci are present [25]. Variable gene dosage *within* the same population has been first postulated in order to account for the well-defined trimodal distribution of percent levels of Hb G $\alpha^{Philadelphia}$ observed in American Black populations by *Milner and Huisman* [18] and *Baine et al.* [5]. *Trabuchet et al.* [29] also proposed variable gene dosage as the cause of the bimodal distribution (31 and 40%) observed for Hb J Mexico ($\alpha^{54\ Gln \to Glu}$) in the Algerian population. According to these authors, the proportion of α-chain variants around means of approximately 20–25, 30–33 and 40–45% can be explained by assuming four, three or two active loci respectively per diploid complement, one of them bearing the amino acid substitution (i.e., $\alpha^X\alpha^A/\alpha^A\alpha^A$, $\alpha^X/\alpha^A\alpha^A$ or $\alpha^X\alpha^A/\alpha^A$, α^X/α^A). *Milner and Huisman* [18] observed in a study of eight families that percentages of Hb G around means of 33% were associated with slightly unbalanced synthetic ratio, while percentages around means of 46% were constantly associated with microcytosis, hypochromia and considerably impaired synthetic balance. Nevertheless, the authors excluded the possibility of a linked α-thalassaemia. These findings would provide evidence that the human α-genes always possess approximately the same quantitative expression, and hence genotypes containing less than four active loci give rise to proportional α-chain deficit, and consequently to haematological pictures resembling the mild forms of α-thalassaemia. The authors also suggest that the α-thalassaemia forms of black Africans could be simply due to both α^A/α^A and $\alpha^A/\alpha^A\alpha^A$ genotypes. However, it remains unclear whether these genotypes necessarily cause a total α-chain deficit. *Baine et al.* [5] in fact, in their study of 53 black American heterozygotes for Hb G $\alpha^{Philadelphia}$ observed the percentage of Hb variant to be negatively correlated with the mean cell volume (MCV) and the mean cell haemoglobin (MCH) content, but no evidence for unbalanced chain synthesis was found in both 30 and 40% Hb G classes. The authors suggest that transcriptional and/or translational control mechanisms limiting the complementary chain production could balance the decreased absolute amount of α-chain synthetized by the lowered α-gene dosage (the α-thalassaemia abnormalities would be due both to diminished α-chain synthesis and absence of the balancing control mechanism(s)).

Moreover, heterozygous and homozygous subjects for Hb J Tongariki ($\alpha^{115\ Ala \to Asp}$) (proposed genotypes α^J/α^A and α^J/α^J respectively [16]) and heterozygotes with 40–45% of Hb Anantharaj ($\alpha^{11\ Lys \to Glu}$) [23] seem to show no haematological or clinical evidence of α-thalassaemia.

All these observations allow to postulate the existence of at least two different haplotypes containing a single active α-chain locus, one which causes unbalanced globin chain synthesis, and the other which seems not to. Then, it is tempting to extrapolate, assuming the α-gene system to be at present

which a glycine had been replaced by an aspartic acid (α^{57} or α^{59}) the abnormal peptides were submitted to sequential degradation and in both cases the identification of the N terminal residue by the dansyl-chloride technique proved the amino acid substitution to be $\alpha^{57\ Gly \to Asp}$ as in Hb Norfolk [4].

Biosynthetic results are reported in table I: all subjects were found to have an overall α/non-α-chain synthesis ratio within the normal range, and moreover the $\alpha^{Norfolk}$/total α-ratios from the five Hb Norfolk carriers appeared in good agreement with the respective proportions of Hb variant.

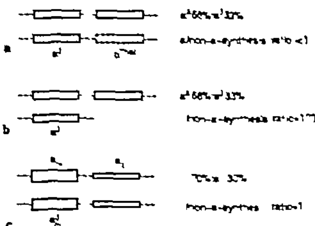


Fig. 2. Three different genic arrangements which account for a level of about 33% of abnormal haemoglobin. For simplicity α-genes are assumed to be linked. Variant genes are shaded. α_M and α_L represent *more* and *less* producing genes (for explanation see text).

Discussion

Structural Studies

In the steric model proposed by *Perutz* [21] for Hb A residue 57 occupies the sixth position in the helical E segment of the α-chain, next to the distal haem-linked histidine residue α^{58} (E7). Nevertheless, the substitution from Gly to Asp occurring at this position in the Hb Norfolk molecule does not appear to influence its stability or its functional properties, at least as far as clinical symptoms are concerned. In addition, it shows no abnormalities in the haem-binding as in the tendency to methaemoglobin formation [24]. It is worth noting that the only other known haemoglobin with a substitution of the same residue, Hb L Persian Gulf ($\alpha^{57\ Gly \to Arg}$) [24] similarly produces no appreciable clinical consequences, and that position E6 shows indeed a wide phylogenetic variability in other known globins [12].

Genetic Considerations

In all Hb Norfolk heterozygous carriers of this family, the father and 4 siblings, the abnormal haemoglobin occurs at a constant level averaging 33%, which can be accounted for on the basis of at least three different hypotheses concerning possible underlying genic arrangements.

(a) According to the 4 α-chain genes model proposed by *Lehmann and Carrell* [14] the observed proportion of abnormal haemoglobin can be explained assuming the presence of two active α^A loci on one chromosome and one α^J locus on the other one, the second locus on this chromosome having been involved in an α-thalassaemia mutation (i.e., the $\alpha^A\alpha^A/\alpha^J\alpha^{thal}$ arrangement, see figure 2a). This hypothesis has been first postulated in order to account for the 33% level of Hb G Philadelphia (α^{68} Asn→Lys) observed in a number of carriers by *French and Lehmann* [13] and later by *Politis Tsegos et al.* [22]. This arrangement is expected to cause a defective α-chain production, which would result in the slight unbalance of the α/non-α-chain synthesis ratio typical

3 Baglioni, C.: An improved method for the fingerprinting of human hemoglobin. Biochim biophys. Acta 48. 392 396 (1961).

4 Baglioni, C. A chemical study of haemoglobin Norfolk. J biol. Chem. 237 69–74 (1962).

5 Baine, R. M., Rucknagel, D. L., Dublin, P A., Jr and Adams, J G Trimodality in the proportion of hemoglobin G Philadelphia in heterozygotes: evidence for heterogeneity in the number of human alpha chain loci. Proc. natn. Acad. Sci. USA 73 3633–3636 (1976).

6 Beaven, G. H Hornabrook, R. W Fox, R H., and Huehns, E. R.. Occurrence of heterozygotes and homozygotes for the α-chain haemoglobin variant Hb J (Tongariki) in New Guinea. Nature, Lond. 235 46–47 (1972).

7 Betke, K., Marti, H. R., and Schlicht, L. Estimation of small percentages of foetal haemoglobin. Nature, Lond. 184 1877–1878 (1959).

8 Cappabianca, M. P Giuliani, A., Marinucci, M and Mavilio, F Thin-layer isoelectric focusing applied to detection of normal hemoglobin fractions. LAB J Res. Lab. Med., IV 563–566 (1977).

9 Carrell, R. W and Kay R. A simple method for detection of unstable haemoglobins Br J Haemat. 23 615–619 (1972).

10 Clegg, J B., Naughton, M. A., and Weatherall, D. J Abnormal human haemoglobins. Separation and characterization of the α- and β-chains by chromatography and the determination of two new variants, Hb Chesapeake and Hb J (Bangkok). J molec. Biol. 19 91 108 (1966).

11 Dacie, J V and Lewis, S. M.. Practical haematology 5th ed. (Churchill, London 1975).

12 Dayhoff, M. O.. Atlas f protein sequence and structure (National Biomedical Research Foundation, Silver Spring, Md. 1972).

13 French, E. A. and Lehmann, H. Is haemoglobin G Philadelphia linked to α-thalassaemia? Acta haemat. 46. 149–155 (1971).

14 Lehmann, H. and Carrell, R. W Differences between α- and β-chain mutants of human haemoglobin and between α- and β-thalassemia. Possible duplication of the α-chain gene. Br med. J iv 748–750 (1968)

15 Marengo-Rowe, A. J Rapid electrophoresis and quantitation of haemoglobin on cellulose acetate. J clin. Path. 18. 790–792 (1965).

16 Marinucci, M., Mavilio, F Tentori, L., and Alberti, R.. Hb O Indonesia (α_2 116 (GH4) Glu→Lys β_2) in association with β-thalassemia. Hemoglobin 2. 59–63 (1978).

17 Mehrotra, T N., Gupta, S. C., Sinha, R., Lehmann, H., and Wiltshire, B. G.. Haemoglobin Norfolk in Nepali Gurkhas. Humangenetik 27 347–349 (1975).

18 Milner P F and Huisman, T H. J Studies on the proportion and synthesis of haemoglobin G Philadelphia in red cells of heterozygotes, homozygote and heterozygote for both haemoglobin G and α-thalassaemia. Br J Haemat. 34 207–220 (1976).

19 Modiano, G., Brancati, C., Marinucci, M., Massa, A., Mavilio, F and Tentori, L. The genetic polymorphism of the globin haplotypes in a population from Calabria (southern Italy). (In preparation, 1978).

20 Nute, P E.. Multiple hemoglobin α-chain loci in monkeys, apes and man. Ann. N.Y Acad Sci. 241 39–60 (1974).

21 Perutz, M. F Structure and function of haemoglobin. I. A tentative atomic model of horse oxyhaemoglobin. J molec. Biol. 13 646–668 (1965).

22 Politis-Tsegos, C.; Lang, A., Stathopoulou, R., and Lehmann, H.. Is haemoglobin G Philadelphia linked to α-thalassaemia? Further evidence. Hum. Genet. 31 67–74 (1976).

23 Pootrakul, S., Kematorn, B., Na-Nakorn, S and Suanpan, S.. A new haemoglobin variant. haemoglobin Anantharaj, alpha 11 (A9) Lysine →Glutamic acid. Biochim. biophys. Acta 405 161 166 (1975).

4 Rahbar S., Kinderlerer J L., and Lehmann, H.. Haemoglobin L Persian Gulf: 57 (E6) Gly→Arg. Acta haemat. 42: 169–175 (1969).

25 Rucknagel, D. L. and Winter W P Duplication of structural genes for hemoglobin α and β chains in man. Ann. N.Y Acad. Sci. 241 80–92 (1974).

26 Shibata, S., Iuchi, I., and Miyaji, T.: Abnormal hemoglobins discovered in Japan. Acta haemat. Jap. 29· 115–127 (1966).

27 Smyth, D. G.. In Methods in enzymology vol XI, p. 214 (Academic Press, New York 1967).

28 Tentori, L., Carta Sorcini, M., and Buccella,

still in course of duplication, that the first have arisen from a duplication of a single α-gene followed by loss of one of the two resulting loci (or loss of its function) while the latter could represent a not yet duplicated α-gene

In the present case the 33% heterozygotes for Hb Norfolk exhibit normal synthetic ratios as well as values of MCV and MCH within the normal range. The three active loci arrangement (i e., $\alpha^J/\alpha^A\alpha^A$ see figure 2b) may be suggested as responsible for the observed percentages of Hb variant. However it must be noted that assuming the subject I 1 to have the $\alpha^J/\alpha^A\alpha^A$ arrangement, each α locus should account for the synthesis of about 5 pg/cell of α-chain (see table I) while in the subject I 2 (assumed genotype $\alpha^A\alpha^A/\alpha^A\alpha^A$) each of the four loci is expected to produce a slightly lesser amount of α-chain (i e. 1/4 of the total α-chain synthetized ≃ 3.9 pg/cell) This means that in the offspring a regulatory mechanism should take place in order to maintain the observed balance of the synthetic ratios and at the same time, a normal level of the total haemoglobin production (the MCH and MCV values are always normal in these subjects)

(c) The third hypothesis assumes each chromosome to have a duplicated gene with unequal expression of each locus (fig 2c) As a matter of fact, quantitative disparities among the haemoglobin chains produced by duplicated α- and non-α-loci appear frequently among primates [20] Convincing evidence for such a situation in man has been furnished by *Trabuchet et al.* [30] in a study of a large Algerian family in which homozygous carriers of Hb J Mexico producing also α^A-chains were present. On the basis of their biosynthetic findings the authors postulated the presence of two α-loci per chromosome, which synthetize different amounts of α-chain and explained the abnormal haemoglobin level of about 33% observed in the heterozygotes assuming the mutation to be placed on the more-producing locus, while the other one in *cis* synthetizes a lesser amount of α^A-chains.

In the present case, this model would be in agreement with all findings. Nevertheless, on the grounds of our limited number of data, we are unable to substantiate convincingly or to reject this temptative hypothesis.

In conclusion it must be pointed out that even though there are now good reasons to believe in the existence of a quantitative polymorphism of the α-chain human haplotypes, we are still unable to determine, for a single case, the exact structural basis underlying a specific phenotypic expression. Conclusive or at least more sound interpretations will be probably furnished by more recent experimental approaches, such as gene dosage by hybridization techniques and DNA and RNA sequencing.

Acknowledgements

The authors are greatly indebted to Dr *G Modiano* for much helpful discussion and for critically reading the manuscript. Thanks are due to Dr *A Massa* for her kind help and advice.

References

1 Abramson, R. K. Rucknagel, D. L., and Shreffler D C. Homozygous Hb J Tongariki. evidence for only one alpha chain structural locus in Melanesians. Science, N Y *169* 194–196 (1970).

2 Ager J A. M., Lehmann, H., and Vella, F Haemoglobin 'Norfolk' a new haemoglobin found in an English family Br med. J *ii*. 539–541 (1958).

Acta haemat. 61: 47–51 (1979)

Inherited Bleeding Syndromes in Jordan

Elias A. K. Alsabti and M. Hammadi
Royal Scientific Society, Amman

Key Words. Inherited bleeding syndromes · Hemophilia · von Willebrand's disease · Afibrinogenemia · Platelet dysfunction

Abstract. This paper presents data on the occurrence and pattern of inherited bleeding syndromes (IBS) in Jordan, a hitherto unexplored problem. In 1978, during the first 12 months of a prospective study at a major medical center, 91 patients from 51 families were diagnosed as having IBS. All patients were referred because of moderate-to-severe bleeding diatheses; they included 52 hemophiliacs, 27 patients with von Willebrand's disease, 4 with hemophilia B (IX-deficit), 2 with afibrinogenemia, 1 with prothrombin deficiency and 4 were thought to have platelet dysfunction. The clinical and laboratory features of the patients observed in Jordan do not seem to be significantly different from those of patients in Western Europe or North America.

Introduction

The incidence of inherited bleeding syndromes (IBS) may be different among different ethnic groups. Hemophilia was thought to be rare among Chinese and Black populations [*Hougie* 1972; *Wintrobe* 1974], whereas factor XI deficiency is reported to be prevalent among Jews in the United States [*Muir and Ratnoff* 1975].

In order to examine the incidence of IBS in a predominantly semitic population with high birth rates and a high incidence of consanguinity, a prospective study was carried out of the occurrence and pattern of IBS in patients admitted to the King Hussein Medical Center, Amman, Jordan, during a period of 12 months.

Material and Methods

Starting March 11, 1977, patients referred because of suspected bleeding disorders were investigated for the purpose of this study. A careful history and family history were obtained and all patients were initially screened with platelet count, prothrombin time (PT), and activated partial thromboplastin (PTT). Abnormalities of any of these tests were further investigated with the necessary laboratory procedure. Clotting factor assays were performed as seemed appropriate. When the clinical or laboratory findings suggested platelet dysfunction, the following procedures were

C., Hemoglobin Abruzzo: α 143 (H21) His→Arg. Clinica chim. Acta 38 258–262 (1972).
29 Trabuchet, G., Dahmane, M., Pagnier J., Lable, D and Benabadji, M.. Hb J Mexico in Algeria. arguments for an heterogeneous distribution of α genes. FEBS Lett. 61 156–158 (1976).
30 Trabuchet, G., Pagnier J., Benabadji, M., and Labie, D.. Homozygous cases for hemoglobin J Mexico (α 54 [E3] Gln→Glu): evidence for a duplicated α gene with unequal expression. Hemoglobin 1 13–25 (1976–77)
31 Wajkman, H. Elian, J et Labie, D.. Stabilité thermique de l'hémoglobine. Intérêt théorique et pratique d'une méthode d'étude cinétique. Nouv Revue fr Hémat. 13 89–94 (1973).
32 Weatherall, D J., Clegg, J B Supa Na Nakorn, and Prawase Wasi The pattern of disordered haemoglobin synthesis in homozygous and heterozygous β-thalassaemia. Br J Haemat. 16. 251–267 (1969).
33 Wilkinson T Kronenberg, H., Beale, D., and Lehmann, H. The second observation of haemoglobin Norfolk occurring in an Italian family together with β-thalassaemia. Med. J Aust. i, 910–912 (1967)

Received. March 16, 1978
Accepted. May 24 1978

Prof. Leonardo Tentori,
Laboratorio di Patologia non Infettiva,
Istituto Superiore di Sanità,
Viale Regina Elena 299
I-00161 Roma (Italy)

Table I. Diagnosis and distribution of patients

Diagnosis	Number of families	Number of patients investigated	Relatives history of bleeding	Total alive bleeders	Relatives with history of fatal hemorrhage
Hemophilia A	25	53	24	77	17
VWD	11	27	19	46	12
Hemophilia B	8	4	3	7	10
Afibrinogenemia	4	2	–		1
Prothrombin deficiency	1	1		1	–
Platelet dysfunction	2	4	1	5	–
Total	51	91	47	128	40

Table II. Age, sex, religion and ethnic origin

Diagnosis	Number of patients investigated	Age, years range (mean + 1 SD)	M/F	Religion (M/C)	Ethnic origin	
					Arab	Assyrian
Hemophilia A	53	0.4–50 (16 ± 0.01)	53/0	53/0	52	1
VWD	27	1.0–32 (15 ± 0.9)	19/8	25/2	27	0
Hemophilia B	4	1.2–13 (6 ± 0.4)	4/0	4/0	3	1
Afibrinogenemia	2	1.8–15 (7.2 ± 0.1)	1/1	1/1	2	0
Prothrombin deficiency	1	2	1/0	1/0	1	0
Platelet dysfunction	4	5–19 (9 ± 0.7)	2/2	4/0	4	0

M/C = Moslem/Christian M/F = male/female.

Table III. Family history (FH) and apparent mode of inheritance

Diagnosis	Number of cases	FH positive	FH negative	Inheritance
Hemophilia	53	45	8	sex-linked
VWD	27	20	7	autosomal dominant (10) autosomal recessive (17)
Hemophilia B	4	3	1	sex-linked
Afibrinogenemia	2	2	0	autosomal recessive
Prothrombin deficiency	1	0	1	autosomal recessive
Platelet dysfunction	4	3	1	autosomal recessive
Total	91	73	18	

performed. bleeding time, clot retraction, platelet coagulant activity (platelet factor 3 release), prothrombin consumption, and examination of platelet morphology by light microscopy If all laboratory studies gave normal or equivocal results but the history was still suggestive of a bleeding diathesis, the patients were reinvestigated at repeated intervals. Available members of the family were also investigated. More than $^3/_4$ of the patients were studied on more than one occasion and most of the severe bleeders had multiple hospital admissions and repeated investigations. Routine laboratory procedures were performed by standard techniques. For coagulation studies venous blood was anticoagulated with $^1/_{10}$ volume of 3.8% trisodium citrate. The PT PTT and one-stage factor XII, XI, IX, VIII, V XIII assays, and two-stage prothrombin assay were performed in duplicates [*Biggs* 1972] Factor-deficient substrate plasma was obtained from Warner Lambert & Co. (Hampshire, England). In a few patients the thromboplastin generation test (TGT) [*Hardisty and Ingram*, 1965] was performed as a screening procedure for factor VIII or IX deficiency Plasma fibrinogen of EDTA-anticoagulated blood was estimated turbidometrically after heat precipitation, as described by *Millar et al* [1971] In the patients with afibrinogenemia, the complete absence of fibrinogen was further documented by immunoelectrophoresis and immunodiffusion (Ouchterlony method) [*Ouchterlony* 1962] using goat anti-human fibrinogen antiserum (Behringwerke, Marburg, FRG) The prothrombin consumption and clot retraction were estimated as described by *Hardisty and Ingram* [1965]. Platelet coagulant activity (platelet factor 3 release) was estimated by modification of a procedure previously described by *Al-Mondhiry et al* [1970] platelet rich plasma was shaken manually for 5 min with 30 μM epinephrine without the use of an aggregometer Modified Ivy bleeding time was performed as described by *Cartwright* [1968] Patients were instructed not to take drugs known to interfere with platelet function for at least 3 days prior to testing. Normal bleeding time, measured by the same technique, in 42 healthy adults was 5.92 ± 1.49 min.

Certain criteria for the diagnosis of hemophilia, von Willebrand's disease (VWD), and platelet dysfunction were used.

Hemophilia (classic, VIII deficiency): (a) reduced factor VIII coagulant activity; (b) normal bleeding time; (c) no clinical or laboratory evidence of an acquired bleeding disorder

Von Willebrand's disease: (a) normal platelet count and morphology; (b) prolonged bleeding time in excess of 12 min, (c) reduced factor VIII level (d) pattern of inheritance. the occurrence of multiple cases involving both sexes satisfying the preceding three criteria (e) evidence of *de novo* synthesis of factor VIII following plasma transfusion (5 patients) [*Shulman*, 1967]

Platelet dysfunction. (a) normal platelet count, (b) normal coagulation profiles including PT PTT and coagulation factor assays (c) prolonged bleeding time in excess of 12 min (d) impaired clot retraction (e) abnormal prothrombin consumption; (f) abnormal platelet coagulant activity (platelet factor 3 release).

Other bleeding disorders were diagnosed by standard *Biggs* [1972] criteria.

Results

91 patients from 51 families were documented to have inherited bleeding disorders (table I) The age, sex, religion and the ethnic origin of the patients are shown in table II Most of the patients live in or around Amman. Data about family history and the apparent mode of inheritance are shown in table III. The apparent mode of inheritance in patients with definite family history of bleeding seem to be sex linked in patients with hemophilia A and B autosomal dominant (10/27) or autosomal recessive (17/27) in patients with VWD It is autosomal recessive for the patients with afibrinogenemia, prothrombin deficiency platelet dys function.

Despite a very high rate of consanguinity among the 51 families studied, no homozygous females were encountered among the hemophilia A and B patients. In some pedigrees the parents and/or grandparents of

Table I. Diagnosis and distribution of patients

Diagnosis	Number of families	Number of patients investigated	Relatives history of bleeding	Total alive bleeders	Relatives with history of fatal hemorrhage
Hemophilia A	25	53	24	77	17
VWD	11	27	19	46	12
Hemophilia B	8	4	3	7	10
Afibrinogenemia	4	2		2	1
Prothrombin deficiency	1	1		1	
Platelet dysfunction	2	4	1	5	
Total	51	91	47	128	40

Table II. Age, sex, religion and ethnic origin

Diagnosis	Number of patients investigated	Age, years range (mean + 1 SD)	M/F	Religion (M/C)	Ethnic origin	
					Arab	Assyrian
Hemophilia A	53	0.4–50 (16 ± 0.01)	53/0	53/0	52	1
VWD	27	1.0–32 (15 ± 0.9)	19/8	25/2	27	0
Hemophilia B	4	1.2 13 (6 ± 0.4)	4/0	4/0	3	1
Afibrinogenemia	2	1.8–15 (7.2 ± 0.1)	1/1	1/1	2	0
Prothrombin deficiency	1	2	1/0	1/0	1	0
Platelet dysfunction	4	5–19 (9 ± 0.7)	2/2	4/0	4	0

M/C = Moslem/Christian M/F = male/female.

Table III. Family history (FH) and apparent mode of inheritance

Diagnosis	Number of cases	FH positive	FH negative	Inheritance
Hemophilia	53	45	8	sex-linked
VWD	27	20	7	autosomal dominant (10) autosomal recessive (17)
Hemophilia B	4	3	1	sex-linked
Afibrinogenemia	2	2	0	autosomal recessive
Prothrombin deficiency	1	0	1	autosomal recessive
Platelet dysfunction	4	3	1	autosomal recessive
Total	91	73	18	

the propositi are maternal and paternal cousins simultaneously indicating homozygosity of recessive or sex-linked disease.

4 patients from 4 families with moderate mucocutaneous bleeding were diagnosed to have platelet dysfunction. It is qualitative platelet defect due to normal coagulation profiles and other criteria previously described. Platelet coagulant activity (platelet factor 3 release) was abnormal in 2 patients and another 2 patients had impaired prothrombin consumption. A more specific diagnosis could not be made in any of these patients due to lack of studies of other aspects of platelet function e.g. platelet aggregation or retention in glass-bead columns. Glanzmann's thrombasthenia is a likely possibility though mild VWD could not be excluded [*Weiss* 1975]

Eight obligate carriers (mothers of affected children 5 hemophiliacs and 2CD) were available for investigations. Factor VIII or IX level ranged from 40% to over 100%. Only 1 young mother with factor VIII level of 65% gave history of brushing and prolonged menstrual bleeding

Bleeding among the hemophilia A and B patients was moderate to severe, mostly multiple arthritic, mucocutaneous, soft tissue, genitourinary and gastrointestinal There was definite correlation between the severity of bleeding and the degree of factor deficiency the most severe bleeders had factor VIII or IX level below 1%, and all of them had levels less than 5%. Patients with VWD had a factor VIII level ranging from 2 to 16%, their bleeding is moderately severe. The bleeding is mild in cases with prothrombin deficiency or platelet dysfunction and mostly mucocutaneous.

Lyophilized concentrated factor VIII is not available for treatment. The cases were treated with whole blood or plasma transfusion.

Conclusion

The clinical and laboratory features were similar to the reported features of European and American patients [*Biggs* 1972 *Hougie* 1972] Although the number of cases is too small to give accurate analysis, hemophilia A and VWD are the most common forms of IBS encountered in this country Comparing the absolute incidence of IBS in this study which is about 2/10 000 population and the relative incidence of the two most common disorders with that of *Biggs* [1972] are about the same. The difference in absolute and relative incidence between this study and that of *Biggs* [1972] can be explained by the fact that in Jordan, only those with moderate to severe bleeding consult physicians. The large number of family members (40 in 51 families) who had fatal hemorrhage attests to the severity of the bleeding problem in the pedigrees investigated. One important factor which influences IBS in Jordan is the high rate of consanguinity prevalent in this country 49 of the 51 families studies (96%) have consanguineous, usually first cousin, marriages. Despite this, however we did not identify any homozygous females among the sex linked disorders, e.g., hemophilia A and B

Acknowledgements

I would like to acknowledge *His Royal Highness Crown Prince Hassan* for his financial support and encouragement. Many thanks to Prof. *A Ersley* for his valuable review of the manuscript.

References

Al-Mondhiry H., Marcus, A. J. and Spaet, T. H.. On the mechanism of platelet function inhibition by acetylsalicylic acid. Proc. Soc. exp. Biol. Med. *133* 532 (1970).

Biggs, R.. Human blood coagulation, hemostasis, and thrombosis, pp. 215, 614 (Blackwell, Oxford 1972).

Cartwright, G. Z.. Diagnostic laboratory hematology p. 367 (Grune & Stratton, New York 1968).

Hardisty R. M. and Ingram, G. L. Bleeding disorders, p. 271 (Blackwell, Oxford 1965).

Hougie, C.. Disorders of hemostasis – Congenital disorders of blood coagulation factors, in Williams *et al.* Hematology p. 1193 (McGraw-Hill, New York 1972).

Millar H. R., *et al.* An evaluation of the heat precipitation method for plasma fibrinogen estimation. J clin. Path. *24* 827 (1971).

Muir, W. A. and Ratnoff, O.: The prevalence of plasma thromboplastin antecedent (PTA, factor XI) deficiency Blood 22. 569 (1974).

Ouchterlony O.: Diffusion in gel method for immunological analysis. II. Prog. Allergy vol. 6, p. 30 (Karger Basel 1962).

Shulman, N. R.. The physiologic basis for therapy of hemophilia (factor VIII deficiency) and related disorders. Ann. Intern., Med. *67* 856 (1967).

Weiss, H. J. Platelet physiology and abnormalities of platelet function. New Engl. J. Med. *293* 580 (1975).

Wintrobe, M. M.. The hereditary coagulation disorders. Clinical hematology (Fed.), p. 1164 (Lea & Febiger Philadelphia 1974).

Received: April 18, 1978
Accepted. July 10, 1978

Dr. Elias Ababti, c/o Mrs. W. Aljaff,
Philharmonic Residence N4, Liverpool L8 7SD
(England)

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

References

Al-Mondhiry H., Marcus, A. J and Spaet, T H.. On the mechanism of platelet function inhibition by acetylsalicylic acid. Proc. Soc. exp. Biol. Med. *133* 532 (1970).

Biggs, R.. Human blood coagulation, hemostasis, and thrombosis, pp. 215, 614 (Blackwell, Oxford 1972).

Cartwright, G. Z.. Diagnostic laboratory hematology p. 367 (Grune & Stratton, New York 1968).

Hardisty R. M. and Ingram, G. L. Bleeding disorders, p. 271 (Blackwell, Oxford 1965).

Hougie, C.. Disorders of hemostasis – Congenital disorders of blood coagulation factors; in Williams *et al.* Hematology p. 1193 (McGraw-Hill, New York 1972).

Millar, H. R., *et al.* An evaluation of the heat precipitation method for plasma fibrinogen estimation. J clin. Path. *24* 827 (1971).

Muir W A. and Ratnoff, O.. The prevalence of plasma thromboplastin antecedent (PTA, factor XI) deficiency Blood *22.* 569 (1974).

Ouchterlony O.: Diffusion in gel method for immunological analysis. II. Prog. Allergy vol. 6, p. 30 (Karger Basel 1962).

Shulman, N. R.: The physiologic basis for therapy of hemophilia (factor VIII deficiency) and related disorders. Ann. Intern., Med. *67* 856 (1967).

Weiss, H. J Platelet physiology and abnormalities of platelet function. New Engl. J Med. *293.* 580 (1975).

Wintrobe, M. M.. The hereditary coagulation disorders. Clinical hematology (Fed.), p. 1164 (Lea & Febiger Philadelphia 1974).

Received. April 18, 1978
Accepted: July 10, 1978

Dr Elias Alsabti, c/o Mrs. W Aljaff,
Philharmonic Residence N4, Liverpool L8 7SD
(England)

Acta haemat. 61: 52–54 (1979)

Amydopyrine Pancytopenia. Detection of Leucocytotoxic Antibodies by a ^{51}Cr-Release Test[1]

M. Jeannet, D. Levy and C. Legendre

Department of Medicine, Hôpital Cantonal, Geneva

Key Words. Amydopyrine pancytopenia · Lymphocytotoxic antibodies · Leukocytotoxic antibodies · ^{51}Cr Release Test

Abstract. Amydopyrine is known to cause agranulocytosis by an immunological mechanism [2]. However pancytopenia due to amydopyrine is extremely rare and usually mild and transient [7]. This report describes a patient with severe pancytopenia presumably due to amydopyrine sensitivity. Granulocytotoxic and lymphocytotoxic antibodies were detected in the patient's serum using a ^{51}Cr-release test.

Case History, Methods and Results

A 50-year-old woman presented with a history of sore throat, chills and malaise, which had not responded to a course of tetracycline. She was febrile and ill with an oral yellow exudate. The leucocyte count was 0.7 × 10⁹/l with total absence of neutrophils; Hb 11 g%, reticulocytes 0.5%, platelet 84 × 10⁹/l. A bone marrow aspirate showed poor cellularity with absence of all granulocytes and reduced erythroid precursors and megacaryocytes. Cultures of the oral exudate grew *Candida albicans*. 3 weeks previously she had taken on several occasions an analgesic containing amydopyrine (Spasmocibalgine) for a severe dental pain. She was started on gentamicin and cloxacillin and nursed with reverse barrier precautions. The following days she remained acutely ill and her pancytopenia became more severe (Hb 7.4 g%, reticulocytes 0%, platelets 14 × 10⁹/l). A new bone marrow aspirate showed only plasmocytes and lymphocytes.

On examination of the eyes white peripapillar exudates were seen strongly suggesting Candida endophtalmitis. Parenteral antibiotics were withdrawn and intravenous amphotericin and 5-fluoro-cytosine were begun. Several granulocyte transfusions were given during the next few days. On this regimen the patient felt gradually better; she became afebrile and her eye lesions began to regress. 15 days after admission her leuco-

[1] This work was supported by the Swiss National Foundation for Scientific Research (Grant No. 3.407.0.74).

Table I. Serum cytotoxicity studies

Days after admission	Serum antibody					
	autologous granulocytes	allogeneic granulocytes	autologous lymphocytes	allogeneic lymphocytes	polymorphonuclear leucocytes 10^9/l	lymphocytes 10^9/l
Day 4	ND	*20.2*[2]	*41.8*[2]	*54.7*[2]	0	714
Day 6	ND	*22.9*	*59.0*	*12 4*	270	670
Day 10	*26.3*	*34.5*	*19.3*	*14.9*	130[3]	760
Day 17	*45.9*	*45 4*	*15.1*	*8.9*	2,000	1,600
Day 20	*40 4*	*41.3*	*5.9*	−1.5	2,262	1,292
Day 148	10.8	0.2	*6.3*	0.9	3,520	2,176

Autologous granulocytes were tested several months after recovery
Percent of specific ¹Cr-release. Statistically significant values are italicized.
Between days 5 and 10 the patient received 5 granulocyte transfusions.

cyte count began to rise, but her anaemia and thrombocytopenia persisted for 2 more weeks. When she was discharged 48 days after admission her leucocyte count was 6.9 × 10^9/l. Serum specimens were tested for granulocyte and lymphocyte cytotoxicity. Granulocyte antibodies were studied by a ^{51}Cr-release cytotoxicity test modified from *Boxer and Stossel* [1]. Lymphocyte antibodies were assayed by the ^{51}Cr-release test of *Rogentine* [5] and by a standard microlymphocytotoxicity test.

A potent cytotoxic granulocyte and lymphocyte antibody was demonstrated in the first serum sample available (before leucocyte transfusions were given) and also in the four subsequent samples (table I). All the active phase serums were cytotoxic for the patient's own cells, isolated several months after recovery. Ten random granulocyte and lymphocyte populations tested were affected by the cytotoxic antibody.

Using the standard micromethod of lymphocytotoxicity the antibody titre was found to be 1 1,024 against the patient's own lymphocytes and 1 8 to 1.32 against random lymphocytes. Both granulocyte and lymphocyte cytotoxic activity were detected in the IgM fractions after serum fractionation using Sephadex A 50.

Comments

Despite many case reports of drug-related agranulocytosis, successful attempts to demonstrate leucocyte antibody have been scanty [4]. Leucocyte agglutination tests were often used, but the results have been difficult to interpret and widely questioned. The present serologic findings using a simple and reliable assay [1] provide strong evidence that auto-antibodies (probably drug induced) specific for neutrophils and lymphocytes were the pathogenetic factor. The aetiologic role of amidopyrine in the pancytopenia appears very likely because of the gradual correction of all symptoms after

withdrawal of the drug and also because of a positive lymphocyte transformation test with amidopyrine. Granulocyte ^{51}Cr-cytotoxicity could be a useful test in suspected amidopyrine sensitivity.

References

1 Boxer, L. A. and Stossel, T. P.: Effects of anti human neutrophil antibodies *in vitro*: quantitative studies. J. clin. Invest. *53:* 1534 (1974).
2 De Gruchy, G. C.: Drug induced blood disorders, p. 78 (Blackwell, Oxford 1975).
3 Flandrin, G.; Daniel, M. T.; Brière, J.; Varet, B.; Pestel, M. et Bernard, J.: L'agranulocytose au pyramidon, une aplasie brève des trois lignées. Nouv. Revue fr. Hémat. *11:* 219 (1971).
4 Magis, C. G.; Barge, A., and Dausset: Serological study of an allergic agranulocytosis due to noramidopyrine. J. clin. exp. Immun. *3:* 989 (1968).
5 Rogentine, G. N.: Histocompatibility testing 1967, p. 371 (Munksgaard, Copenhagen 1967).

Received: April 21, 1978
Accepted: July 10, 1978

Dr. med. M. Jeannet, Unité d'Immunologie de Transplantation, Hôpital Cantonal,
Rue Micheli-du-Crest 24, CH–1211 Genève (Switzerland)

Acta haemat. 61 55-56 (1979)

The Preparation of ^{59}Fe-Labelled Transferrin of High Radioactivity for Ferrokinetic Studies on Small Laboratory Animals

J. Holá, J. Vácha and J. Boháček

Institute of Biophysics, Czechoslovak Academy of Sciences, Brno

Key Words. Purification of ^{59}Fe-labelled transferrin · Sephadex gel filtration · Ion exchanger Dowex 1 X-4 · Ferrokinetic studies · Small laboratory animals

Abstract. A modified method of Cavill for the separation of the complex of isotopically labelled iron and transferrin from unbound iron residues is described. The method employing an ion exchanger permits to obtain preparations with a high radioactivity per volume unit of solution, which is necessary for carrying out ferrokinetic studies on small laboratory animals.

As emphasized by *Cavill* [1971] the preparation of a complex of isotopically labelled iron with transferrin using fresh plasma requires the removal of the residue of unbound iron, if the preparation is to be really pure. This author described a method of purification by means of an anion exchange resin, which is intended for clinical purposes and requires the application and withdrawal of large quantities of plasma from the experimental subject. However working with small laboratory animals requires that preparations of high radioactivity per volume unit be obtained. In the present article, a suitable modification of the method is described, together with the necessary details not included in the above-cited work.

For the experiments intact mice of strain C57Bl/10ScSnPh aged 3 months were used, the iron content in the diet amounting to 321 ± 30 mg Fe/kg. Blood was taken under ether narcosis from the axillary vessels into glass test tubes without using any anticoagulants (to allow coagulation of fibrin). Samples were centrifuged at 1,400 g for 20 min, the serum was collected and incubated at 37 °C for 60 min in various volume ratios with commercial solution of isotopically labelled ferrous citrate (^{59}F-citrate, of Rotop, GDR, pH 4-6, specific activity 5.47 Ci/g F [2.02 10^{14} Bq/g F], 10.5 µg Fe/1 cm supplied in an isotonic buffer containing ascorbic and citric acid, with 0.9% of benzyl alcohol). The preparate was adjusted to pH 7 by means of 1 N NaOH. An ion exchanger Dowex 1 X-4 was used for separation and Sephadex gel filtration (G 75) was used to verify efficiency of separation.

The preparation of the anion exchange resin Dowex-1 X-4 (100-200 mesh) included the follow-

ing: before pouring into the column, the anion exchange resin was allowed to swell in distilled water and minor particles were removed by decantation. Ion exchanger was transferred to the OH form by the action of 3 *N* NaOH for 30 min under continuous stirring. After it had been thoroughly washed with water and a neutral reaction had been obtained, the anion exchange resin was transformed to the Cl form by stirring with 3 *N* HCl, also for 30 min. After washing with distilled water until a neutral reaction was obtained, the anion exchange resin was poured into a column formed by a 2 cm^3 plastic syringe (i.d. 10 mm, height 20 mm). The required amount of serum incubated with ^{59}Fe was applied on the column thus prepared. As the amount of serum retained in the column was irrelevant for our purposes, the column was not washed at all during the separation, to avoid dilution of the serum. (If however further use of the column is intended, it should naturally be rinsed in distilled water after separation is completed.)

The purity of the preparation was checked by separation on a Sephadex G-75 column prepared as follows: before use, Sephadex G 75 Medium (Pharmacia, Uppsala) was allowed to swell in distilled water for 24 h and was then washed with distilled water in a beaker so that the poorly sedimenting fraction could be separated by decanting. Then it was filled into a column formed by a pipette with an internal diameter of 8 mm to a height of 180 mm. Purified serum in amount of 50 mm^3 was applied to the Sephadex column and the column was washed out with saline solution. The radioactivity of the eluate flowing out of the column was measured in 1 cm^3 fractions on a Nuclear Chicago Mark I gamma spectrometer in the region of 0.5–1.4 MeV (efficiency 19%) and radioactivity levels were plotted depending on the volume passed through the column. The curves obtained were compared with the analogous curves corresponding to the incubated plasma not purified on the Dowex, which had only been separated on the Sephadex column after incubation.

From a comparison of the curves it follows that, with a 1.2 volume ratio of ^{59}Fe-citrate solution and serum (Fe concentration 10.5 µg Fe/cm^3) about 20% activity remains unbound after a 1 h incubation. This unbound iron is, however completely bound by the ion exchanger. A ratio of the solution of ^{59}Fe and serum of 1 1 or 1 4 proved to be less effective. The radioactivity of the preparation per 0.1 cm^3 amounts to about 5.10^5 impulses/min (with a 19% efficiency of measurement), which is a level sufficient for ferrokinetic studies on small laboratory animals. Consequently purified serum may be used immediately (without any further purity check by means of Sephadex) for intravenous application to experimental animals, provided the above-described separation procedure on the Dowex 1 X-4 is followed. The preparation can be stored at 10 °C for 24 h, or it can be slightly concentrated by freeze-drying. In recommended procedures defibrinated serum (but not plasma) must be used to prevent precipitation during passage through the column.

References

Cavill, I. The preparation of ^{59}Fe-labelled transferrin for ferrokinetic studies. J clin. Path. *24* 472–474 (1971)

Received April 18, 1978
Accepted July 7 1978.

J Holá, Institute of Biophysics,
Královopolská 135 612 65 Brno,
(Czechoslovakia)

Correspondence

Acta haemat. 61 57–58 (1979)

Spurious Macrocytic Anemia

To the Editor

Macrocytosis often accompanied by anemia is usually due to folic acid or vitamin B_{12} deficiency liver disease or alcoholism. In the absence of these pathological conditions and in the presence of cold agglutinins, erroneous macrocytic values can be produced by the Coulter Counter

A 64-year-old male was admitted to the Department of Medicine A because of pneumonia. On admission the temperature was 37.6 °C, the blood pressure 160/100 mm Hg, and auscultatory findings were consistent with right lower lobe pneumonia. The relevant laboratory data were: sedimentation rate of 40/60 mm (Westergren), hemoglobin 12.8 g/dl, hematocrit 38%, RBC 1.8×10^{6}/µl, mean corpuscular volume 176 fl, mean corpuscular hemoglobin (MCH) 70 pg and mean corpuscular hemoglobin concentration (MCHC) 40% Similar values were obtained in 8 consecutive blood counts. The peripheral blood smear showed marked erythrocyte agglutination. The white blood cell count (WBC) was 10,300/µl with slight shift to the left, and the platelet count was 120,000/µl. Cultures of the sputum were negative and no acid fast bacilli were seen on direct smear Serum anti-I cold agglutinins in titer of 1:64 were demonstrated. The patient was successfully treated with tetracycline and cotrimoxazole and was discharged on the 10th day of hospitalization

Spurious macrocytic anemia has been described in very few patients all of whom had serum cold agglutinins [1–4] The mechanism by which these agglutinins cause this phenomenon is readily explained by the technique of the Coulter Counter

Anti-I cold reactive antibodies produce particles with increased volume by agglutinating erythrocytes. The counter considers each microagglutinate as one cell, and thus less RBCs are being recorded each with a higher volume, 1.8×10^{6}/µl and 176 fl, in the patient described. Accordingly high MCH and MCHC values are calculated. The clue to diagnosis of spurious macrocytic anemia is the presence of high values of MCH and MCHC (> 36%) together with disproportionally high hemoglobin and hematocrit values.

This case as well as those previously reported, illustrate the phenomenon of 'spurious macrocytic anemia' and emphasize that, in its presence, serum cold agglutinins should be looked for

References

1 Corbérand, J. Boneu, B., Pris, J. Larrard, B. de et Colombies, P. Indices érythrocytaires

ing: before pouring into the column, the anion exchange resin was allowed to swell in distilled water and minor particles were removed by decantation. Ion exchanger was transferred to the OH form by the action of 3 *N* NaOH for 30 min under continuous stirring. After it had been thoroughly washed with water and a neutral reaction had been obtained, the anion exchange resin was transformed to the Cl^- form by stirring with 3 *N* HCl, also for 30 min. After washing with distilled water until a neutral reaction was obtained, the anion exchange resin was poured into a column formed by a 2 cm^3 plastic syringe (i.d. 10 mm, height 20 mm) The required amount of serum incubated with ^{59}Fe was applied on the column thus prepared. As the amount of serum retained in the column was irrelevant for our purposes, the column was not washed at all during the separation, to avoid dilution of the serum. (If, however further use of the column is intended, it should naturally be rinsed in distilled water after separation is completed.)

The purity of the preparation was checked by separation on a Sephadex G 75 column prepared as follows before use, Sephadex G-75 Medium (Pharmacia, Uppsala) was allowed to swell in distilled water for 24 h and was then washed with distilled water in a beaker so that the poorly sedimenting fraction could be separated by decanting. Then it was filled into a column formed by a pipette with an internal diameter of 8 mm to a height of 180 mm. Purified serum in amount of 50 mm was applied to the Sephadex column and the column was washed out with saline solution. The radioactivity of the eluate flowing out of the column was measured in 1 cm^3 fractions on a Nuclear Chicago Mark I gamma spectrometer in the region of 0.5–1 4 MeV (efficiency 19%) and radioactivity levels were plotted depending on the volume passed through the column. The curves obtained were compared with the analogous curves corresponding to the incubated plasma not purified on the Dowex, which had only been separated on the Sephadex column after incubation.

From a comparison of the curves it follows that, with a 1.2 volume ratio of ^{59}Fe-citrate solution and serum (Fe concentration 10.5 μg Fe/cm^3) about 20% activity remains unbound after a 1 h incubation. This unbound iron is, however completely bound by the ion exchanger A ratio of the solution of ^{59}Fe and serum of 1.1 or 1 4 proved to be less effective. The radioactivity of the preparation per 0 1 cm^3 amounts to about 5 10^4 impulses/min (with a 19% efficiency of measurement), which is a level sufficient for ferrokinetic studies on small laboratory animals. Consequently purified serum may be used immediately (without any further purity check by means of Sephadex) for intravenous application to experimental animals, provided the above-described separation procedure on the Dowex 1 X-4 is followed. The preparation can be stored at 10 C for 24 h, or it can be slightly concentrated by freeze-drying. In recommended procedures defibrinated serum (but not plasma) must be used to prevent precipitation during passage through the column.

References

Cavill, I. The preparation of ^{59}Fe-labelled transferrin for ferrokinetic studies. J clin. Path. *24* 472–474 (1971)

Received. April 18, 1978
Accepted. July 7 1978.

J Holá, Institute of Biophysics,
Královopolská 135 612 65 Brno,
(Czechoslovakia)

Correspondence

Acta haemat. 61: 57–58 (1979)

Spurious Macrocytic Anemia

To the Editor

Macrocytosis often accompanied by anemia is usually due to folic acid or vitamin B_{12} deficiency, liver disease or alcoholism. In the absence of these pathological conditions and in the presence of cold agglutinins, erroneous macrocytic values can be produced by the Coulter Counter.

A 64-year-old male was admitted to the Department of Medicine A because of pneumonia. On admission the temperature was 37.6 °C, the blood pressure 160/100 mm Hg, and auscultatory findings were consistent with right lower lobe pneumonia. The relevant laboratory data were: sedimentation rate of 40/60 mm (Westergren), hemoglobin 12.8 g/dl, hematocrit 32%, RBC $1.8 \times 10^6/\mu l$, mean corpuscular volume 176 fl, mean corpuscular hemoglobin (MCH) 70 pg and mean corpuscular hemoglobin concentration (MCHC) 40%. Similar values were obtained in 8 consecutive blood counts. The peripheral blood smear showed marked erythrocyte agglutination. The white blood cell count (WBC) was 10,300/µl with slight shift to the left, and the platelet count was 120,000/µl. Cultures of the sputum were negative and no acid fast bacilli were seen on direct smear. Serum anti-I cold agglutinins in titer of 1:64 were demonstrated. The patient was successfully treated with tetracycline and cotrimoxazole and was discharged on the 10th day of hospitalization.

Spurious macrocytic anemia has been described in very few patients all of whom had serum cold agglutinins [1–4]. The mechanism by which these *agglutinins* cause this phenomenon is readily explained by the technique of the Coulter Counter.

Anti-I cold reactive antibodies produce particles with increased volume by agglutinating erythrocytes. The counter considers each microagglutinate as one cell, and thus less RBCs are being recorded each with a higher volume, $1.8 \times 10^6/\mu l$ and 176 fl, in the patient described. Accordingly high MCH and MCHC values are calculated. The clue to diagnosis of spurious macrocytic anemia is the presence of high values of MCH and MCHC (> 36%) together with disproportionally high hemoglobin and hematocrit values.

This case, as well as those previously reported, illustrate the phenomenon of spurious macrocytic anemia and emphasize that, in its presence, serum cold agglutinins should be looked for.

References

1 Corbérand, J.; Bourra, B.; Pris, J.; Larrard, B. de et Colombies, P.: Indices érythrocytaires

ing: before pouring into the column, the anion exchange resin was allowed to swell in distilled water and minor particles were removed by decantation. Ion exchanger was transferred to the OH form by the action of 3 *N* NaOH for 30 min under continuous stirring. After it had been thoroughly washed with water and a neutral reaction had been obtained, the anion exchange resin was transformed to the Cl form by stirring with 3 *N* HCl, also for 30 min. After washing with distilled water until a neutral reaction was obtained, the anion exchange resin was poured into a column formed by a 2 cm³ plastic syringe (i.d. 10 mm, height 20 mm). The required amount of serum incubated with ^{59}Fe was applied on the column thus prepared. As the amount of serum retained in the column was irrelevant for our purposes, the column was not washed at all during the separation, to avoid dilution of the serum. (If however further use of the column is intended, it should naturally be rinsed in distilled water after separation is completed.)

The purity of the preparation was checked by separation on a Sephadex G 75 column prepared as follows: before use, Sephadex G 75 Medium (Pharmacia, Uppsala) was allowed to swell in distilled water for 24 h and was then washed with distilled water in a beaker so that the poorly sedimenting fraction could be separated by decanting. Then it was filled into a column formed by a pipette with an internal diameter of 8 mm to a height of 180 mm. Purified serum in amount of 50 mm³ was applied to the Sephadex column and the column was washed out with saline solution. The radioactivity of the eluate flowing out of the column was measured in 1 cm³ fractions on a Nuclear Chicago Mark I gamma spectrometer in the region of 0.5–1.4 MeV (efficiency 19%), and radioactivity levels were plotted depending on the volume passed through the column. The curves obtained were compared with the analogous curves corresponding to the incubated plasma not purified on the Dowex, which had only been separated on the Sephadex column after incubation.

From a comparison of the curves it follows that, with a 1:2 volume ratio of ^{59}Fe-citrate solution and serum (Fe concentration 10.5 μg Fe/cm³) about 20% activity remains unbound after a 1 h incubation. This unbound iron is, however completely bound by the ion exchanger. A ratio of the solution of ^{59}Fe and serum of 1:1 or 1:4 proved to be less effective. The radioactivity of the preparation per 0.1 cm³ amounts to about 5.10⁶ impulses/min (with a 19% efficiency of measurement), which is a level sufficient for ferrokinetic studies on small laboratory animals. Consequently purified serum may be used immediately (without any further purity check by means of Sephadex) for intravenous application to experimental animals, provided the above-described separation procedure on the Dowex 1 X-4 is followed. The preparation can be stored at 10 °C for 24 h, or it can be slightly concentrated by freeze-drying. In recommended procedures defibrinated serum (but not plasma) must be used to prevent precipitation during passage through the column.

References

Cavill, I. The preparation of ^{59}Fe-labelled transferrin for ferrokinetic studies. J. clin. Path. 24 472–474 (1971)

Received. April 18, 1978
Accepted. July 7 1978.

J. Holá, Institute of Biophysics,
Královopolská 135 612 65 Brno,
(Czechoslovakia)

Acta haemat. 61 59 (1979)

Oxacillin-Induced Granulocytopenia

Sir

I cannot agree with *Fallon et al.* [1] that there is no published literature reporting the occurrence of granulocytopenia secondary to oxacillin therapy. Probably the first case of oxacillin-induced neutropenia was reported by *Freedman* [2] in January 1965 in a 33-year-old woman following 2½ months of therapy with oral oxacillin, 3 g/day. Without undertaking an extensive literature search, I can cite at least 13 cases of granulocytopenia, leukopenia and neutropenia (including one geriatric case and 8 pediatric cases) where oxacillin was implicated as the causative agent [3–7]. In the majority of cases, oxacillin was used in rather high dosages to treat several types of infections (e.g. septic arthritis, epidural abscess, wound infections, osteomyelitis, bacteremia, endocarditis) caused primarily by *Staphylococcus aureus*. The abnormalities were uniformly reversible when therapy with the antimicrobial agent was discontinued. The duration of exposure to oxacillin (2 days to 10 weeks) and the rapidity with which the patients' conditions returned to normal (2 days to 4.5 weeks) varied widely.

As a postscript, I must add that a recent paper by *Arbeter et al.* [8] presented at the 88th Annual Meeting of the American Pediatric Society and Society for Pediatric Research, 26–28 April, 1978, reported three additional cases of oxacillin-induced neutropenia.

I hope that the above information will be of interest to the authors under reference and also to the readers of your esteemed journal.

References

1 Fallon, J. A., Tall, A. R., Janis, M. G., and Brauer, M. J.: Oxacillin-induced granulocytopenia. Acta haemat. 59: 163–170 (1978).
2 Freedman, M. A.: Oxacillin – apparent hematologic and hepatic toxicity. Rocky Mt. med. J. 62: 34–36 (1965).
3 Ahern, M. J.; Hicks, J. E., and Andriole, V. T.: Neutropenia during high dose intravenous oxacillin therapy. Yale J. biol. med. 49: 351–360 (1976).
4 Lavanthal, J. M. and Silken, A. B.: Oxacillin-induced neutropenia in children. J. Pediat. 89: 769–771 (1976).
5 Chu, J. Y.; O'Connor, D. M., and Schmidt, R. R.: The mechanism of oxacillin-induced neutropenia. J. Pediat. 90: 668–669 (1977).
6 Brook, L.: Leukopenia and granulocytopenia after oxacillin therapy. South. med. J. 70: 565–566 (1977).
7 Ortbals, D. W. and Marr, J. J.: Profound neutropenia caused by oxacillin. South. med. J. 70: 1245–1246 (1977).
8 Arbeter, A. M.; Tolpin, M. D.; Piercy, S. A.; Yachetti, S. D.; Danish, M. S.; Warren, W. S., and Plotkin, S. A.: Nafcillin-oxacillin comparative clinical trial. Pediat. Res. 12: 459 (1978).

J. B. Ghosh,
811 Clifford Avenue,
Ardmore, PA 19003 (USA)

This letter was presented to one of the authors concerned (Dr. *M. J. Brauer*) who refrained from further comment.

The Editor

aberrants donnés par le Coulter Counter modèle S chez les malades présentant des agglutinines froides. Path. Biol., Paris *21* 549–553 (1973).
2 Hattersley P G., Gerard, P W Caggiano, V., and Nash, D R. Erroneous values on the Model S Coulter Counter due to high titer cold autoagglutinins. Am. J clin. Path. *55* 442–446 (1971).
3 Lange, J A. de; Eernisse, G J., and Veltkamp, J J.. Cold agglutinins and the Coulter Counter Model S. Am. J clin. Path. *58* 599–600 (1972).
4 Petrucci, J V., Dunne, P A., and Chapman, C. C. Spurious erythrocyte indices as measured by the Model S Coulter Counter due to cold agglutinins. Am. J clin. Path. *56*. 500–502 (1971).

Received March 20, 1978
Accepted. August 3, 1978

R. Tur Kaspa and Y Levo
Department of Medicine A,
Hadassah University Hospital,
Jerusalem (Israel)

Original Papers

Acta haemat. *61*: 61–67 (1979)

The Use of 75Se-Methionine as a Tracer of Thrombocytopoiesis

I. *In vivo* Incorporation of the Tracer into Platelet Proteins: a Biochemical Study

E. Dassin and Y. Najean

Service Central de Médecine Nucléaire, Hôpital Saint Louis, Paris

Key Words. Thrombocytopoiesis · Kinetics · 75Se methionine · Platelet actomyosin

Abstract. This study was undertaken to define to which platelet components 75Se-methionine is bound after its injection to normal rats, and to study the curves of specific radioactivity of each labelled fraction. It has been shown that, for a part, platelet labelling *is due to adsorption of plasma proteins (albumin and fibrinogen) after their synthesis.* For another part, methionine is directly incorporated in the bone marrow precursors into platelet actomyosin, a protein constituent of the cells; the variations of the specific radioactivity of this component indicate that 75Se-methionine can be used as a cohort label of platelets.

Introduction

An indirect measurement of the effective production of platelets can be deduced from the determination of their mean life-span, after *in vitro* labelling with $Na_2{}^{51}CrO_4$ or DF32P; direct information has also been deduced from morphological studies of bone marrow megakaryocytes and experiments using 3H-thymidine infusion. However these methods only give incomplete informations on megakaryocytic maturation and platelet production, and do not appear convenient enough for physiological investigation on megakaryocytopoiesis in man and animals.

For some years, 75Se-labelled methionine has been proposed as a marker of megakaryocytic line, in animals for a quantification of thrombopoietic substances [*Penington,* 1971; *Levin et al.,* 1971; *Evatt et al.,* 1974] as well as for identification of platelet production defects in man [*Penington,* 1969; *Ardaillou and Najean,* 1969; *Brodsky et al.,* 1970; *McIntyre et al.,* 1970]. However, it has been clearly shown [*Penner,* 1966; *Ardaillou et al.,* 1971] that this tracer cannot be considered as an ideal marker of cohort of cells: not only is its uptake by the platelet line low compared to that to other blood cell lines, but also the permanent utilization of the labelled circulating proteins results in a permanent new labelling of platelets even after the initially marked cohort of cells has disappeared.

We have then tried to define to which

Varia

Association Française des Hémophiles

The French Association of Haemophiliacs International Prize of FF 15,000, whose aim is to encourage medical research into the disease, will be awarded for the third time in July 1979

The previous prize winners have been Dr *J P Allain* (La Queue-lez Yvelines, France) in 1975 and Dr *R. H Wagner* (Chapel Hill, N C., USA) in 1977

The regulations in French and English will be forwarded on request by the Secretariat of the selection committee (Association Française des Hémophiles, CNTS, 6 rue Alexandre-Cabanel, F 75015 Paris, France).

The work submitted for the prize must reach the Secretariat of the selection committee by March 15th 1979 at the latest.

3rd Meeting of the Mediterranean Blood Club

April 11–15 1979
Antalya, Turkey

This joint Meeting will be together with the Mediterranean Blood Club and the Turkish Society of Haematology and supported by the Antalya Medical Faculty

Addresses for correspondence:
Dr Orhan N Ulutin, Levent, Begonya Sok. 6
Istanbul, Turkey and
Congress Bureau,
VIP Turizm Pirinççioğlu Ltd.,
Cumhuriyet Cad. 12,
Elmadağ, Istanbul (Turkey).

Original Papers

Acta haemat. 61: 61–67 (1979)

The Use of ^{75}Se-Methionine as a Tracer of Thrombocytopoiesis

I. *In vivo* Incorporation of the Tracer into Platelet Proteins: a Biochemical Study

E. Dassin and Y. Najean

Service Central de Médecine Nucléaire, Hôpital Saint-Louis, Paris

Key Words. Thrombocytopoiesis · Kinetics · ^{75}Se-methionine · Platelet actomyosin

Abstract. This study was undertaken to define to which platelet components ^{75}Se-methionine is bound after its injection to normal rats, and to study the curves of specific radioactivity of each labelled fraction. It has been shown that, for a part, platelet labelling is due to adsorption of plasma proteins (albumin and fibrinogen) after their synthesis. For another part, methionine is directly incorporated in the bone marrow precursors into platelet actomyosin, a protein constituent of the cells; the variations of the specific radioactivity of this component indicate that ^{75}Se-methionine can be used as a cohort label of platelets.

Introduction

An indirect measurement of the effective production of platelets can be deduced from the determination of their mean life-span, after *in vitro* labelling with $Na_2{}^{51}CrO_4$ or DF^{32}P; direct information has also been deduced from morphological studies of bone marrow megakaryocytes and experiments using ^{3}H-thymidine infusion. However, these methods only give incomplete informations on megakaryocytic maturation and platelet production, and do not appear convenient enough for physiological investigation on megakaryocytopoiesis in man and animals.

For some years, ^{75}Se-labelled methionine has been proposed as a marker of megakaryocytic line, in animals for a quantification of thrombopoietic substances [*Penington* 1971; *Levin et al.* 1971; *Evatt et al.* 1974] as well as for identification of platelet production defects in man [*Penington* 1969; *Ardaillou and Najean* 1969; *Brodsky et al.* 1970; *McIntyre et al.*, 1970]. However, it has been clearly shown [*Penner* 1966; *Ardaillou et al.* 1971] that this tracer cannot be considered as an ideal marker of cohort of cells: not only is its uptake by the platelet line low compared to that to other blood cell lines; but also the permanent utilization of the labelled circulating proteins results in a permanent new labelling of platelets even after the initially marked cohort of cells has disappeared.

We have then tried to define to which

components of the platelets radio-methionine was bound after its injection to normal rats. The present paper demonstrates that the tracer is, for a part, adsorbed to the circulating platelets as labelled plasma proteins (albumin and fibrinogen) and for another part, incorporated into a protein constituent of the platelets. A following paper will present the evolution in function of time of the specific radioactivity of every labelled component, thus demonstrating that [75]Se-methionine can be really used as a cohort label of the platelet line.

Material and Methods

Male Wistar rats weighing between 200 and 300 g were used. [75]Se-methionine (spec. act. 30–70 mCi/mg Commissariat à l'Energie Atomique Saclay France) was injected into the tail vein at a dose of 20 μCi/100 g body weight. [35]S-sulfate was injected at a dose of 100 μCi/100 g.

Blood samples were obtained by cardiac puncture under ether anaesthesia using plastic syringes containing acidified citrate solution: trisodium citrate, 4.48 g; citric acid, 2.73 g; anhydrous *D*-glucose, 2.00 g; distilled water 100 ml. 1 vol of anticoagulant was used for 9 vol of blood.

The samples were then centrifuged twice at 400 *g* for 10 min. Platelet rich plasma was centrifuged at 1,500 *g* for 20 min, and the platelet button was washed twice in 0.154 *M* NaCl containing 10% acidified citrate solution. In every experiment, we have checked to ensure that the platelet suspension was not contaminated: haemoglobin in lysates was not detectable by spectrophotometry; no leucocytes were visible by optical microscopy.

Isolation of Sulfated Mucopolysaccharides from Blood Platelets

Mucopolysaccharides were extracted from the platelet pellet obtained 3 days after *in vivo* [75]Se-methionine administration or [35]S-sulfate infusion, by papain digestion at 65 C during 4 h with slow stirring (papain: 1/50) according to the method published by *Olsson and Gardell* [1967] using cetyl pyridinium chloride. The isolated fraction was resuspended in n-propanol, counted and stained for mucopolysaccharides using the specific stain described by *Bitter and Muir* [1962].

Isolation of Soluble Platelet Proteins

Preparation of the extract: the platelet pellet was resuspended in 0.15 *M* Tris-HCl pH 7.5 and fragmented by freezing and thawing 4 times in liquid nitrogen. The homogenate was then centrifuged at 28,000 *g* for 30 min to remove intact platelets and debris.

1 ml of 20% trichloroacetic acid (TCA) was added to 1 ml of the supernatant. The precipitate was isolated, washed twice with 10% TCA, and its radioactivity was measured.

Gel filtration of the supernatant was carried out at 4 C with Sephadex G 200 (Pharmacia, Uppsala, Sweden) using a column 35 cm long and 2.5 cm in diameter; elution was performed at 4 °C with 0.1 *M* Tris-HCl pH 7.5 1 *M* NaCl, at a rate of 15 or 20 ml/h. Spectrophotometric absorption at 280 nm and the radioactivity of each 3.5-ml eluate fraction were measured.

Fibrinogen was identified by clotting with thrombin. Thrombin (2 U/ml) was added slowly to the first protein peak isolated from Sephadex chromatography. The samples were incubated at 37 C for 30 min and the clot was removed with a glass rod, washed with distilled water and dissolved in alkaline urea (6.7 *M* urea, 0.2 *M* NaOH). The optical density at 280 nm was measured on the supernatant of the samples, and the radioactivity was measured in the clots.

Platelet Actomyosin Extraction

This was based on the technique described by *Nachman et al.* [1967]. Fresh platelet pellets, obtained by centrifugation at 28,000 *g* for 30 min, were solubilised with n-butanol for 24 h at 4 °C. After warming to room temperature for 1 h, the tubes were centrifuged at 28,000 *g* for 30 min. Chromatography of the supernatant was carried out with Sephadex G 200, using for elution 0.015 *M* Tris-HCl pH 7.4 containing 0.6 *M* KCl, in a column 35 cm long and 2.5 cm in diameter. The main protein fraction thus obtained was collected and concentrated by Aquacide II (Calbiochem, San Diego, Calif.), then dialysed against 500 ml of

0.03 *M* Tris-HCl pH 7.5 which causes precipitation due to fall in ionic strength and loss of Mg^{++} ions. This precipitate was isolated by centrifugation at 100,000 g for 3 h, and its radioactivity was determined.

Immunodiffusion

Immunodiffusion using Ouchterlony plates was carried out with rabbit antibodies against rat plasma (Hyland Lab., Los Angeles, Calif.) and the protein fractions were isolated from the platelet homogenates.

Radioactivity Measurement

^{75}Se radioactivity was measured in well scintillation counter with an accuracy of ± 2%. The observed counts were related to the total radioactivity of the studied sample and also to the quantity of protein isolated, as measured by optical density (OD). The specific radioactivity was the radioactivity measured per 1 unit of OD for each protein. ^{35}S was measured by liquid scintillation with an accuracy of ± 3%.

Table I. Isolation of platelet mucopolysaccharides after digestion by papain of platelet homogenates[1]

	Radioactivity %		Mucopolysaccharide stain
	^{75}Se	^{35}S	
Dialysate	0	0	negative
Stroma	10 ± 5	59 ± 8	
Proteins and glycoproteins	85 ± 10	0	negative
Mucopolysaccharides	0	30 ± 10	positive

Results are given as the percentage of the radioactivity present in the different fractions, compared with those of the platelet button before papain digestion (mean of 10 rats ± 1 SD).

Results

Incorporation of the Tracers into Platelet Mucopolysaccharides

As shown in table I no radioactivity due to ^{75}Se is found in the mucopolysaccharidic fraction at the time when platelet radioactivity is maximal. On the other hand, all the ^{35}S-sulfate is found in isolated mucopolysaccharides and in fragments of membranes not digested by papain.

Incorporation into Proteins Soluble in Tris HCl Buffer at pH 7.4

Absence of free methionine is demonstrated as (1) no radioactivity was found in the dialysate after overnight dialysis against 0.8 m*M* NaCl, at 4 °C of the platelet supernatant (2) 85 % of the platelet supernatant radioactivity is precipitated by TCA 10% while the free methionine remains in suspension.

Sephadex G-200 chromatography of the *in vivo* labelled platelet supernatant enables to separate consistently three different protein fractions (fig. 1a). Radioactivity is only found in fractions I and II. The elution profile differs from that of the plasma samples (fig. 1b).

Fraction I is precipitated by anti-rat plasma antiserum (fig. 2). Its specific radioactivity decreases as rapidly as that of circulating fibrinogen ($t_{1/2}$ = 3 days, fig. 3). Thrombin clots 93 ± 5% of the protein(s) eluted in fraction I, and 82 ± 10% of the radioactivity found in this fraction.

Fraction II migrates chromatographically as the fraction of plasma albumin does. A clear line of precipitation is observed on immunodiffusion plates with anti-rat plasma antiserum (fig.). The specific radioactivity of the fraction decreases slowly ($t_{1/2}$ = 11 days, fig. 3) like *in vivo* labelled circulating albumin. These results suggest that ^{75}Se methionine in this fraction is bound to albumin.

components of the platelets radio-methionine was bound after its injection to normal rats. The present paper demonstrates that the tracer is, for a part, adsorbed to the circulating platelets as labelled plasma proteins (albumin and fibrinogen) and, for another part incorporated into a protein constituent of the platelets. A following paper will present the evolution, in function of time, of the specific radioactivity of every labelled component, thus demonstrating that [75]Se methionine can be really used as a cohort label of the platelet line

Material and Methods

Male Wistar rats weighing between 200 and 300 g were used. [75]Se-methionine (spec. act. 30–70 mCi/mg: Commissariat à l'Energie Atomique, Saclay France) was injected into the tail vein at a dose of 20 μCi/100 g body weight. [35]S-sulfate was injected at a dose of 100 μCi/100 g.

Blood samples were obtained by cardiac puncture under ether anaesthesia using plastic syringes containing acidified citrate solution: trisodium citrate, 4.48 g; citric acid, 2.73 g; anhydrous D glucose, 2.00 g; distilled water 100 ml. 1 vol of anticoagulant was used for 9 vol of blood.

The samples were then centrifuged twice at 400 g for 10 min. Platelet-rich plasma was centrifuged at 1,500 g for 20 min, and the platelet button was washed twice in 0.154 M NaCl containing 10% acidified citrate solution. In every experiment, we have checked to ensure that the platelet suspension was not contaminated: haemoglobin in lysates was not detectable by spectrophotometry no leucocytes were visible by optical microscopy

Isolation of Sulfated Mucopolysaccharides from Blood Platelets

Mucopolysaccharides were extracted from the platelet pellet obtained 3 days after in vivo [75]Se methionine administration or [35]S-sulfate infusion, by papain digestion at 65 C during 4 h with slow stirring (papain: 1/50) according to the method published by *Olsson and Gardell* [1967] using cetyl pyridinium chloride. The isolated fraction was resuspended in n-propanol, counted and stained for mucopolysaccharides using the specific stain described by *Bitter and Muir* [1962].

Isolation of Soluble Platelet Proteins

Preparation of the extract: the platelet pellet was resuspended in 0.15 M Tris-HCl pH 7.5 and fragmented by freezing and thawing 4 times in liquid nitrogen. The homogenate was then centrifuged at 28,000 g for 30 min to remove intact platelets and debris.

1 ml of 20% trichloroacetic acid (TCA) was added to 1 ml of the supernatant. The precipitate was isolated, washed twice with 10% TCA, and its radioactivity was measured.

Gel filtration of the supernatant was carried out at 4 C with Sephadex G 200 (Pharmacia, Uppsala, Sweden) using a column 35 cm long and 2.5 cm in diameter: elution was performed at 4 °C with 0.1 M Tris-HCl pH 7.5 1 M NaCl, at a rate of 15 or 20 ml/h. Spectrophotometric absorption at 280 nm and the radioactivity of each 3.5-ml eluate fraction were measured.

Fibrinogen was identified by clotting with thrombin. Thrombin (2 U/ml) was added slowly to the first protein peak isolated from Sephadex chromatography The samples were incubated at 37 °C for 30 min and the clot was removed with a glass rod washed with distilled water and dissolved in alkaline urea (6.7 M urea 0.2 M NaOH). The optical density at 280 nm was measured on the supernatant of the samples, and the radioactivity was measured in the clots

Platelet Actomyosin Extraction

This was based on the technique described by *Nachman et al.* [1967] Fresh platelet pellets, obtained by centrifugation at 28,000 g for 30 min, were solubilised with n-butanol for 24 h at 4 °C. After warming to room temperature for 1 h, the tubes were centrifuged at 28,000 g for 30 min. Chromatography of the supernatant was carried out with Sephadex G 200, using for elution 0.015 M Tris-HCl pH 7.4 containing 0.6 M KCl, in a column 35 cm long and 2.5 cm in diameter The main protein fraction thus obtained was collected and concentrated by Aquacide II (Calbiochem, San Diego, Calif.), then dialysed against 500 ml of

0.03 *M* Tris-HCl pH 7.5 which causes precipitation due to fall in ionic strength and loss of Mg^{++} ions. This precipitate was isolated by centrifugation t 100,000 g for 3 h, and its radioactivity was determined.

Immunodiffusion

Immunodiffusion using Ouchterlony plates was carried out with rabbit antibodies against rat plasma (Hyland Lab., Los Angeles, Calif.), and the protein fractions were isolated from the platelet homogenates.

Radioactivity Measurements

^{75}Se radioactivity was measured in well scintillation counter with an accuracy of ± ^{0}/$_{0}$. *The observed counts were related to the total* radioactivity of the studied sample and lso to the quantity of protein isolated, as measured by optical density (OD). The specific radioactivity was the radioactivity measured per 1 unit of OD for each protein. ^{35}S was measured by liquid scintillation with an accuracy of ± 3%.

Table I. Isolation of platelet mucopolysaccharides after digestion by papain of platelet homogenates

	Radioactivity %		Mucopoly-saccharide
	^{75}Se	^{35}S	stain
Dialysate	0	0	negative
Stroma	10 ± 5	59 ± 8	
Proteins and glycoproteins	85 ± 10	0	negative
Mucopolysaccharides	0	30 ± 10	positive

Results are given as the percentage of the radioactivity present in the different fractions, compared with those of the platelet button before papain digestion (*mean of 10 rats ± 1 SD*).

Results

Incorporation of the Tracers into Platelet Mucopolysaccharides

As shown in table I, no radioactivity due to ^{75}Se is found in the mucopolysaccharidic fraction at the time when platelet radioactivity is maximal. On the other hand, all the ^{35}S-sulfate is found in isolated mucopolysaccharides and in fragments of membranes not digested by papain.

Incorporation into Proteins Soluble in Tris HCl Buffer at pH 7.4

Absence of free methionine is demonstrated as (1) no radioactivity was found in the dialysate after overnight dialysis against 0.8 m*M* NaCl, at 4 C of the platelet supernatant, (2) 85% of the platelet supernatant radioactivity is precipitated by TCA 10% while the free methionine remains in suspension.

Sephadex G-200 chromatography of the *in vivo* labelled platelet supernatant enables to separate consistently three different protein fractions (fig. 1a). Radioactivity is only found in fractions I and II. The elution profile differs from that of the plasma samples (fig. 1b).

Fraction I is precipitated by anti-rat plasma antiserum (fig. 2). Its specific radioactivity decreases as rapidly as that of circulating fibrinogen (t – 3 days, fig. 3). Thrombin clots 93 ± 5 / of the protein(s) eluted in fraction I, and 82 ± 10% of the radioactivity found in this fraction.

Fraction II migrates chromatographically as the fraction of plasma albumin does. A clear line of precipitation is observed on immunodiffusion plates with anti-rat plasma antiserum (fig. 2). The specific radioactivity of the fraction decreases slowly ($t_{½}$ = 11 days fig. 3) like *in vivo* labelled circulating albumin. These results suggest that ^{75}Se-methionine in this fraction is bound to albumin.

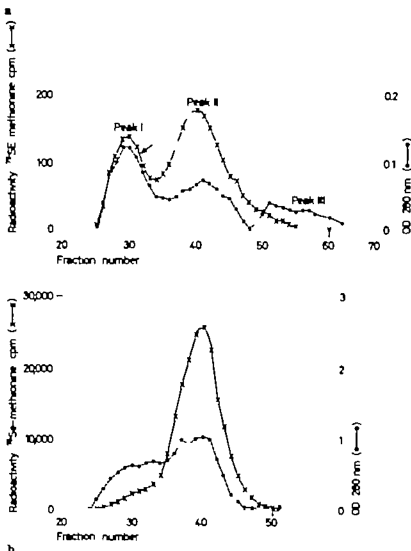


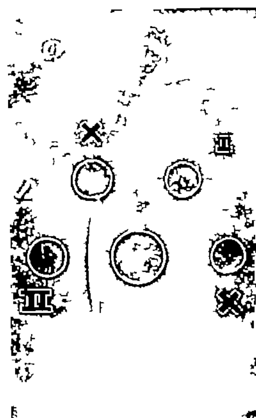
Fig. 1 Vertical chromatography on Sephadex G-200 gel of platelet supernatant, obtained from blood specimens taken 24 h after injection of ⁷⁵Se-methionine (a) and of plasma obtained from the same blood specimens (b) using a column 35 cm long and 2.5 cm in diameter elution with 0.1 *M* Tris-HCl pH 7.5 1 *M* NaCl at a rate of 15–20 ml/h at 4 C.

Incorporation of ⁷⁵Se-Methionine into n Butanol Extractable Protein

A protein of high molecular weight (of the order of 500 000 daltons according to Sephadex migration) and called protein X on the immunodiffusion figure, has been isolated from platelet homogenate It does not precipitate on immunodiffusion in the presence of anti-rat plasma antiserum and the curve of its specific radioactivity in function of time, after ⁷⁵Se methionine infusion, clearly differs from that of the circulating proteins (fig. 2) If the ionic strength is lowered and the Mg ions are eliminated by dialysis, complete precipitation occurs, suggesting that it is platelet actomyosin.

Discussion

For several years, ⁷⁵Se-methionine has been proposed as a cohort tracer for blood cell populations, particularly platelets. The choice of this tracer was determined by various practical and radiobiological considerations such as the easily measurable emission of ⁷⁵Se and the lesser radiobiological hazard than when using long-living β-emitters.



^{75}Se-methionine has been considered as a cohort label of the platelet cell line enabling to measure an autologous platelet life-span and to appreciate, from its incorporation, the degree of an hyper or hypo-production of platelets. However this opinion has been controverted, since the platelet radioactivity curves drawn after *in vivo* infusion of ^{75}Se-methionine in man and animals always show a tail after the time at which the initially labelled cells have disappeared [*Najean and Ardaillou* 1969]. For this reason it appears that a precise determination of the biochemical site of radioactivity uptake was to be done.

The present study was undertaken to define to which platelet component ^{75}Se-methionine is bound, after *in vivo* infusion.

^{75}Se-methionine disappears very rapidly from the circulation and reappears within a

Fig. 2. Ouchterlony immunodiffusion. Fractions I and II (isolated from the supernatant after congelation and thawing): 500 and 600 µg/ml, respectively; fraction X (isolated from the platelet homogenate): 400 µg/ml.

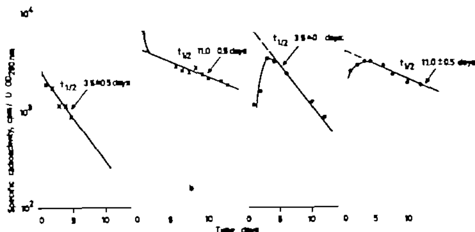


Fig. 3. Changes in specific radioactivity with time. Plasma proteins (×): fibrinogen (a) and albumin (b). Platelet fractions isolated by vertical chromatography (●): fraction I (c) and fraction II (d).

few hours bound to the plasma proteins, mainly albumin [*Awwad et al* 1967] It is well known that albumin is adsorbed on the platelet surface [*Behnke* 1968] most probably on specific sites [*Marcus et al* 1966] Fraction II isolated from platelet supernatant migrates as rat albumin and the evolution of its radioactivity follows the same slope after equilibration as that of the plasma albumin radioactivity

The fibrinogen found in the platelet residue may be, at least for a part a protein synthesised *de novo* by the megakaryocytes [*Soria et al* 1976] but other authors think that platelet fibrinogen is circulating protein adsorbed to the cell membrane [*Karaca et al* 1971] Our observations, especially that of significant radioactivity as soon as the 24th h in fraction I of the soluble platelet proteins (which are partly or wholly fibrinogen) and the fact that the specific radioactivity of this fraction decreases like that of circulating fibrinogen, show that the adsorption phenomenon explains the labelling of this fraction.

We have been able to isolate a platelet protein fraction which has the same molecular weight and solubility as platelet actomyosin. This protein is rich in methionine, and represents 15–20% of the platelet proteins [*Booyse and Rafelson* 1971] It is interesting that the curve of its specific radioactivity (fig. 4) corresponds with that expected from a flash labelling of the marrow precursors and the release of a cohort of cells with a finite life-span the mean life-span calculated from this curve agrees with the platelet survival directly measured by *in vitro* ^{51}Cr labelling and reinfusion [*Odell et al* 1955 *Aster* 1967 *Ginsburg and Aster* 1969] At the 4th day after tracer infusion, the radioactivity incorporated into the specific

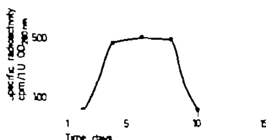


Fig. 4. Specific radioactivity of the platelet actomyosin against time, after infusion of ^{75}Se-methionine in rats.

cell protein was in normal rats approximately equal to the radioactivity adsorbed to the cell membrane as albumin and fibrinogen.

The present study demonstrates that ^{75}Se-methionine binds to the platelets by two different ways. For one part, it is adsorbed to the cell wall as constituent of the plasma proteins (albumin and fibrinogen) labelled from hepatic synthesis the permanent reutilization of methionine for this synthesis and the permanent adsorption of plasma proteins explains the radioactivity observed during more than 15 days in the experimental curves. For another part, methionine is incorporated into specific protein(s), chiefly platelet actomyosin and could then be considered as a true cohort label of the cells.

Acknowledgements

We are grateful to Mrs. *F Danon* service d'Immunochimie, Hôpital Saint Louis, and to *A J Barrett* Leukaemia Research Fund Fellow for their help

References

Ardaillou, N et N Jean, Y Cinétique de la thrombopoïèse utilisant la ^{75}Se-méthionine. Nouv Revue fr Hémat 9 889–895 (1969)

Ardaillou, N N jean, Y and Eberlin, A. Study of platelet kinetics using ⁷⁵Se-selenomethionine, In Paulus, Platelet kinetics, chapt. 13, pp. 131 143 (North Holland, Amsterdam 1971).
Aster R. H Utilization of platelets in rats and man. Blood *30*. 860 (1967).
Awwad, H. K Adelstein, S. J Potchen, E. J and Dealy J B.. The interconversion and reutilization of injected ⁷⁵Se-selenomethionine in the rat. J biol. Chem. *242*. 492 500 (1967).
Behnke, O Electron microscopical observations on the surface coating of human blood platelets. J Ultrastruct Res. *24* 51–69 (1968).
Bitter T and Muir H. M.. A modified uronic acid carbazole reaction. Analyt. Biochem. *4* 330–334 (1962)
Booyse, F M and Rafelson, M. E.. Human platelet contractile proteins: location, properties and function. Ser Haemat. *4* 152 174 (1971).
Brodsky L, Siegel, N H Kahn, S. B Ross, E M and Petkov G Simultaneous fibrinogen and platelet survival with (⁷⁵Se) selenomethionine in man studies in diseases with normal coagulation and in hepato-cellular disease with abnormal coagulation Br J Haemat. *18* 341–355 (1970).
E att, B L. and Levin, J Measurement of thrombopoiesis in rabbits using ⁷⁵Selenomethionine J clin. Invest 48. 1615–1626 (1969).
Evatt, B. L., Shreiner D P and Levin, J Thrombopoietic activity of fractions of rabbit plasma: studies in rabbits and mice J Lab. clin Med. *85*. 364–371 (1974).
Ginsberg, A. D and Aster R. H.. Kinetic studies with ⁵¹chromium-labeled cohort in rats J Lab clin. Med. *74* 138–144 (1969).
Karaca, M., Nilson, I. M and Hedner U Quantitative determination of platelet fibrinogen. J Lab. clin Med *77* 485–489 (1971)
Levin, J., Shreiner D P and Evatt, B. L. Measurement of thrombopoiesis using ⁷⁵Se-selenomethionine in Paulus, Platelet kinetics, chapt. 12, pp 126–132 (North Holland, Amsterdam 1971).
Marcus, A. J Zucker-Franklin, C., Safier L. B., and Ullman, H L. Studies on human platelet granules and membranes. J clin. Invest. *45* 1 28 (1966).
McIntyre, P A Evatt, B Hodkinson, B., and Scheffel, U Selenium-75 seleno-methionine as label for erythrocytes, leukocytes and platelets in man. J Lab. clin. Med. *75* 472–480 (1970)
Nachman, R. L., Marcus, A. J and Safier L. B Platelet thrombosthenin: subcellular localization and function. J clin. Invest. *46* 1380–1389 (1967).
Najean, Y and Ardaillou, N.. The use of ⁷⁵Se-methionine for the *in vivo* study of platelet kinetics. Scand. J Haematol. *6*. 395–401 (1969).
Odell, T T Tausche, F G and Furth, J Platelet lifespan as measured by transfusion of isotopically labeled platelets into rats. Acta haemat *13* 45–52 (1955).
Olsson, I. and Gardell, S.. Isolation and characterization of glycosaminoglycans from human leukocytes and platelets. Biochim. biophys Acta *141* 348–357 (1967)
Penington, D G Assessment of platelet production with ⁷⁵Se-selenomethionine. Br med. J *iv* 782–784 (1969).
Penington, D G Bioassay f thrombopoietin Assay in mice using ⁷⁵Se-selenomethionine; in Paulus, Platelet kinetics, chapt. 14, pp. 143–157 (North Holland, Amsterdam 1971)
Penner J A *Investigation of erythrocyte turnover with selenium-75 labeled methionine.* J Lab. clin Med. *67* 427–438 (1966).
Salmon, J Absorption de protéines par les plaquettes sanguines. Experientia *16* 26–27 (1960).
Soria, J Soria, C., Samama, M Pourot, E., and Kling, C Human platelet fibrinogen: protein different from plasma fibrinogen Path. Biol., Paris *24* suppl pp 15–17 (1976).

Received. April 14, 1978
Accepted: July 11, 1978

E. Damm, Attachée de Recherches,
Institut National de la Santé et
de la Recherche Médicale,
Hôpital Saint-Louis, F–75475
Paris Cédex 10 (France)

Acta haemat. *61* 68–74 (1979)

A Controlled Study of the Effect of Sulfinpyrazone on Platelet Survival and on Platelet-Bound ^{14}C-Serotonin Release in Patients with Previous Myocardial Infarction

M. Cortellaro, C. Boschetti, G. Fassio, M. Basagni and E. E. Polli

Clinica Medica Ia dell'Università and Cattedra di Istituzioni di Matematica per Medici dell'Università, Milano

Key Words. Sulfinpyrazone · Platelet survival · Platelet serotonin release · Hyperbetalipoproteinemia · Myocardial infarction

Abstract. Preliminary data obtained in the ambit of Anturan Reinfarction Italian Study (ARIS) show that, in postmyocardial infarction, reduced platelet survival time occurred in hyperbetalipoproteinemic patients treated with placebo, but not in the group of patients treated with sulfinpyrazone (interaction between treatment and lipemic level is at $p \cong 0.1$). The sulfinpyrazone effect on platelet survival is probably related to the release reaction inhibition as suggested by our *ex vivo* results on platelet bound ^{14}C serotonin release.

Signs of platelet hyperactivity in ischemic cardiopathy have been evidenced in numerous studies [8, 10, 16, 17, 19, 29, 30]. Platelet survival time seems to be one of the most reliable indices of such hyperactivity and of the hypercoagulability which may be found in some thromboembolic states [11, 22, 24, 25, 28]. In May 1976, a multicenter controlled clinical study (Anturan Reinfarction Italian Study, ARIS) [6] was started on the prevention of myocardial reinfarction by sulfinpyrazone, with a follow up of 24 months. The number of patients admitted to the trial is expected to be 1 000 and its end is forecasted for 1980. The aims of the trial are to ascertain firstly if sulfinpyrazone (at a dose of 800 mg/die) prevents fatal and nonfatal myocardial reinfarction and cardiac death, and secondly if any therapeutic efficacy of sulfinpyrazone is correlated with a measurable effect on platelet function as monitored by tests for platelet survival, circulating platelet aggregates, platelet bound ^{14}C-serotonin release, platelet retention, platelet aggregation by ADP and collagen in serial concentrations, and plasmatic heparin-neutralizing activity.

The present paper reports the preliminary results on platelet survival time determined in the 5th month after the episode of infarction pain in 2 balanced subsamples of 10 patients each, 1 treated with sulfinpyrazone and the other with a placebo. Sulfinpyrazone's known efficacy in inhibiting pla

telet release reaction *in vitro* was not demonstrated until recently in *ex vivo* studies. We wanted in our investigation to verify whether with special techniques sulfinpyrazone's effect on platelet survival could be shown *ex vivo* to be related to the release reaction inhibition. The platelet-bound ^{14}C serotonin release by different collagen concentrations was therefore studied in another balanced sample of 10 patients from the ARIS series, 5 treated with sulfinpyrazone and 5 with placebo

Materials and Methods

Platelet Survival Time

The series of 20 patients with previous myocardial infarction consists of 12 patients without clinical evidence of generalized atherosclerosis, diabetes or hypertension and without anomalies in the lipidic pattern, and 8 patients with associated hyperbetalipoproteinemia

No medication other than sulfinpyrazone or placebo was taken by the patients being studied. Each patient showed more than one lipoprotein electrophoretic pattern. None of the patients with hyperbetalipoproteinemia presented hypothyroidism, biliary obstruction or multiple myeloma. 5 normal volunteers (3 men and 2 women) who had taken no medication in the previous 15 days or longer and who had normal concentrations of total and low-density lipoproteins, cholesterol and triglycerides were used as control group. The characteristics of the myocardial patients with normolipoproteinemia and hyperbetalipoproteinemia and of the control subjects are shown in table I.

Platelet survival was performed with ^{75}Se-selenomethionine [3]; survival time was calculated by measuring the interval between 50% of the maximum radioactivity in the anabolic phase and 50% of the maximum radioactivity in the catabolic phase of the curve. Venous blood was obtained from fasting patients. Plasma lipoprotein electrophoresis, cholesterol and triglycerides were measured by automated techniques in routine hospital laboratory use.

In addition to the descriptive statistics (mean,

Table I. Platelet survival time: patients clinical data

Drug	Sex fM		Age	Cholesterol mg/dl	Triglycerides mg/dl	Total lipid mg/dl
Sulfinpyrazone	7	mean	53.90	255.20	153.56	848.33
(= 10)	(70%)	SD	10.78	70 57	38.78	284 80
		range	34–65	120–356	100–225	640–1,570
Placebo	8	mean	49 40	231.00	157.67[1]	787 44
(n = 10)	(80%)	SD	7 31	57 15	102.98	220 04
		range	36–57	130–282	50–396	550–1 182
Subsample	NS		F = 1 18	F = 0.71	F = 0.01	F = 0.71
Total	15	mean	51 65	243.10	155 61	837 94
group	(75%)	SD	9.30	63 77	75 51	252.25
(n = 20)		range	34–65	120–356	50–396	550–1,570
Control	3	mean	52 20	205.00	110.60	
group	(60%)	SD	7 85	16.20	17.87	
(= 5)		range	42–63	185–220	85–130	

fM = Number of males.
One case omitted

Acta haemat 61 68–74 (1979)

A Controlled Study of the Effect of Sulfinpyrazone on Platelet Survival and on Platelet-Bound ^{14}C-Serotonin Release in Patients with Previous Myocardial Infarction

M Cortellaro C Boschetti G Fassio M Basagni and E E Polli

Clinica Medica Ia dell'Università and Cattedra di Istituzioni di Matematica per Medici dell'Università, Milano

Key Words. Sulfinpyrazone Platelet survival Platelet serotonin release Hyperbetalipoproteinemia Myocardial infarction

Abstract Preliminary data obtained in the ambit of Anturan Reinfarction Italian Study (ARIS) show that, in postmyocardial infarction reduced platelet survival time occurred in *hyperbetalipoproteinemic patients treated with placebo but not in the group of patients* treated with sulfinpyrazone (interaction between treatment and lipemic level is at $p \approx 0.1$). The sulfinpyrazone effect on platelet survival is probably related to the release reaction inhibition as suggested by our *ex vivo* results on platelet bound ^{14}C serotonin release.

Signs of platelet hyperactivity in ischemic cardiopathy have been evidenced in numerous studies [8 10 16 17 19 29 30] Platelet survival time seems to be one of the most reliable indices of such hyperactivity and of the hypercoagulability which may be found in some thromboembolic states [11 22, 24 25 28] In May 1976 a multicenter controlled clinical study (Anturan Reinfarction Italian Study ARIS) [6] was started on the prevention of myocardial reinfarction by sulfinpyrazone, with a follow up of 24 months The number of patients admitted to the trial is expected to be 1 000 and its end is forecasted for 1980 The aims of the trial are to ascertain firstly if sulfinpyrazone (at a dose of 800 mg/die) prevents fatal and nonfatal myocardial reinfarction and cardiac death and secondly if any therapeutic efficacy of sulfinpyrazone is correlated with a measurable effect on platelet function as monitored by tests for platelet survival, circulating platelet aggregates, platelet bound ^{14}C serotonin release, platelet retention, platelet aggregation by ADP and collagen in serial concentrations, and plasmatic heparin neutralizing activity

The present paper reports the preliminary results on platelet survival time determined in the 5th month after the episode of infarction pain in 2 balanced subsamples of 10 patients each 1 treated with sulfinpyrazone and the other with a placebo Sulfinpyrazone's known efficacy in inhibiting pla

meters considered (table I). The 5 control subjects had cholesterol and triglyceride levels lower than the 20 infarction patients. The statistical comparison of platelet survival times (table II, fig. 1) shows that there is a nearly significant interaction ($p \simeq 0.1$) between treatment and lipemic level, while there are no significant differences between the rows or between the columns. The row and column mean values are all very similar to that of control subjects. The interaction can be explained by observing that in the hyperlipemic patients group the cell average (treatment effect) moves away from the control group in the opposite direction.

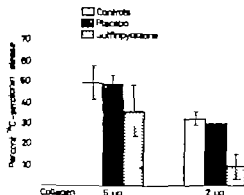


Fig. 2. *Ex vivo* effect of sulfinpyrazone on platelet-bound [14]C-serotonin release by two different concentrations of collagen.

Platelet-Bound [14]C Serotonin Release

The mean value of the [14]C-serotonin release in patients and controls (2 concentrations of collagen) are reported in table III and figure 2. The mean age in the 2 treatment subsamples is slightly different but not in a significant way.

Statistical comparison of the release values shows a highly significant difference between the treatment groups ($p < 0.01$) and between the collagen doses ($p < 0.001$), but no significant interaction is seen. The individual comparisons between treatments made separately for the 2 doses of collagen show significant differences between sulfinpyrazone and controls ($p < 0.001$) and between sulfinpyrazone and placebo ($p < 0.05$ for 5 µg; $p < 0.01$ for 2 µg), but no significant differences are seen between placebo and controls.

Table III. Patients clinical data and platelet-bound [14]C-serotonin release results

Drug		Age	Percent 5-HT release by collagen	
			5 µg	2 µg
Sulfinpyrazone	mean	57.20	35.60	8.60
(n = 5)	SD	9.31	1[illegible].50	5.81
	range	45–66	16–50	4–18
Placebo	mean	46.00	48.60	29.60
(n = 5)	SD	8.43	3.85	3.[illegible]
	range	35–57	44–52	27–34
Total	mean	51.60	42.10	19.10
group	SD	10.24	11.09	11.92
(n = 10)	range	35–66	16–52	4–34
Control	mean	51.50	49.00	32.20
group	SD	7.47	8.25	[illegible].95
(n = 5)	range	42–60	40–56	28–35

Comparison groups $F = 16.28$ ($p < 0.01$); doses $F > 99$ ($p < 0.001$); interaction $F = .22$ (NS).

Discussion

Isolated cases are reported in the literature [22] of coronary artery disease presenting reduced platelet survival not correlated with the lipidic pattern or with adhesivi-

standard deviations), subsample homogeneity was performed by means of the F test and Fisher test. Comparison between survival time in the treat ment samples, classified in 2 unbalanced subsam ples, normolipemic and hyperlipemic patients, was made by means of a suitable analysis of variance [2]

Serotonin Labelling and Release

The method described by *Zucker and Peterson* [31] was used. Collagen (Hormo) was used at 5 and 2 μg/0.8 ml of platelet rich plasma (PRP) Blood was drawn from fasting subjects using a two-syr inge technique. The blood was mixed with 0.1 vol of 3.8% sodium citrate in polypropylene tubes centrifuged at 40 rpm for 15 min at room temperature to yield PRP The platelet count of PRP was 300,000 / l.

In the group of the 10 patients examined, myocardial infarction had also occurred 5 months previously 5 normal volunteers who had not tak en any nonsteroidal anti-inflammatory drugs for at least 10 days prior to the study were used as a control group The clinical data of patients and the control subjects are shown in table III

Overall comparisons were made of the means of collagen-release serotonin in the 3 samples (sul finpyrazone, placebo, controls) and for the 2 doses of collagen (2 and 5 / g) using the split plot analy sis of variance The errors of estimate obtained in this analysis were also used for comparisons be tween treatment pairs at each dose of collagen with the procedure suggested by *Cochran and Cox* [5]

Results

Platelet Survival Time

The two treatment subsamples are homogeneous for age, sex and the lipemic para-

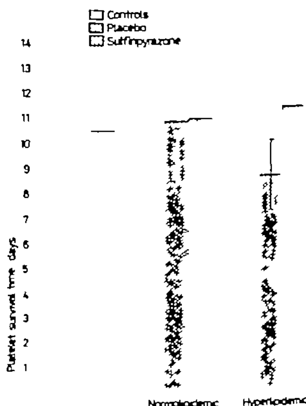


Fig 1 Effect of sulfinpyrazone on platelet survival time.

Table II. Effect of sulfinpyrazone on platelet survival time (days)

Drug	Normol pidemic			Hyperlipidemic			Total group		
	f	mean	SD	f	mean	SD	f	mean	SD
Sulfinpyrazone	6	10.87	1 95	4	11 43	1 03	10	11.09	1 60
Placebo	6	10 90	2.37	4	8 68	1 43	10	11 01	_27
Total group	12	10.88	2.07	8	10.05	1.87	20	11.05	1.91
Controls							5	10 48	1.80

Comparison rows (treatment) F = 1 76 (NS) columns (lipemic level) F = 1.00 (NS) interaction F = _99 ($p \simeq 0.1$)

f = Number of cases.

tion Italian Study (ARIS). 1st Florence Conference on Haemostasis and Thrombosis 1977

7 Cortellaro, M., Fumio, G., Boschetti, C., Basagni, M., and Polli, E. E. Controlled ex vivo inhibiting effect of sulfinpyrazone on platelet function (in press).

8 Entrecknap, P. B. Gooding, P. G., Lansley, T. S., and Avis, P. R. P. Platelet size and function in ischaemic heart disease. J. Atheroscler. Res. 10 41–49 (1969).

9 Genton, E., Gent, M. Hirsh, J. and Harker, L. A. Platelet inhibiting drugs in the prevention of clinical thrombotic disease. New Engl. J. Med. 293 1296–1300 (1975).

10 Gormsen, J. Nielsen, J. D. and Andersen, L. A. Platelet aggregation in patients with ischaemic heart disease, transient cerebral ischaemia and peripheral thrombo-atherosclerosis. Thrombos. Haemost. 38. 32 (1977).

11 Harker, L. A. and Finch, C. A. Thrombokinetics in man. J. clin. Invest. 48 963–974 (1969).

12 Harker, L. A. and Schlichter, S. J. Platelet and fibrinogen consumption in man. New Engl. J. Med. 287 999–1005 (1972).

13 Johm, F. and Tremblay, F. Platelet reactions and immune processes. II. The inhibition of platelet aggregation by complement inhibitors. Thromb. Diath. haemorrh. 22. 466–481 (1969).

14 Kaegi, A., Pineo, G. F. Shimizu, A., Trivedi, H., Hirsh, J. and Gent, M. Arteriovenous shunt thrombosis prevention by sulfinpyrazone. New Engl. J. Med. 290 304–306 (1974).

15 Murphy, E. A. and Mustard, J. F. Coagulation tests and platelet economy in atherosclerotic and control subjects. Circulation 25 114–125 (1962).

16 O'Brien, J. R. Etherington, M., Jamieson, S., Klaber, M. R. and Lincoln, S. V. Platelet function long after arterial and venous thrombosis. Thromb. Diath. haemorrh. 31 279–290 (1974).

17 O'Brien, J. R. and Jamieson, S. A relationship between platelet volume and platelet number. Thromb. Diath. haemorrh. 31 363–365 (1974).

18 Packam, M. A. Warrior, E. S. Glynn, M. E., Senyi, A. S. and Mustard, J. F. Alteration of the response of platelets to surface stimuli by pyrazol compounds. J. exp. Med. 126. 171 191 (1967).

19 Polli, E. E., Cortellaro, M., Frattola, L., Randazzo, A., Candelise, L., Pogliani, E.; Polini, A., Bassi, S., Scotti, G., and Santambrogio, S. Effect of aspirin and dipyridamole on platelet function and on neurological evaluation of patients affected by stroke or transient ischaemic attacks (Springer. Berlin 1977).

20 Ritchie, J. L. and Harker, L. A. Platelet and fibrinogen survival in coronary atherosclerosis. Response to medical and surgical therapy. Am. J. Cardiol. 39 595–598 (1977).

21 Shattil, S. J. Bennett, J. S., Colman, R. W. and Cooper, R. A. Abnormalities of cholesterol phospholipid composition in platelets and low density lipoproteins of human hyperbetalipoproteinemia. J. Lab. clin. Med. 89 341 353 (1977).

22 Steele, P. P. Weily, H. S. Davies, H., and Genton, E. Platelet function studies in coronary artery disease. Circulation 48 1194–1200 (1973).

23 Steele, P. P. Weily, H., and Genton, E. Effect of sulfinpyrazone on platelet survival adhesion and aggregation. Clin. Res. 21 863 (1973).

24 Steele, P. P. Weily, H., Davies, H. and Genton, E. Platelet survival in patients with rheumatic heart disease. New Engl. J. Med. 290 537–539 (1974).

25 Steele, P. P. Weily, H. Davies, H. Pappas, G. and Genton, E. Platelet survival time following aortic valve replacement. Circulation 51 358–362 (1975).

26 Steele, P. P. Battock, D. and Genton, E. Effects of clofibrate and sulfinpyrazone on platelet survival time in coronary artery disease. Circulation 52 473–476 (1975).

27 Sullivan, J. M. Harken, D. E., and Gorlin, R. Pharmacologic control of thromboembolic complication of cardiac valve replacement. New Engl. J. Med. 279 576–580 (1968).

28 Weily, H. S. Steele, P. P. Davies, H., Pappas, G. and Genton, E. Platelet survival in patients with substitute heart valves. New Engl. J. Med. 290 534–537 (1974).

29 Yamazaki, H. Sano, T. Asano, T. and Hidaka, H. Hyperaggregability of platelets in thromboembolic disorders. Thrombos. Haemost. 36 479–482 (1976).

ty aggregation tests, and this suggests the existence of primitively activated platelets. However the majority of authors agree that there is a reduced platelet survival associated with anomalies in the lipidic pattern and/or signs of diffused atherosclerosis [15 20 25 26]

Platelet survival time is a measure of the interaction of platelets with the arterial surface Therefore, in the presence of diffuse atherosclerotic lesions, platelet surface interactions occur to an extent to bring about platelet consumption which can be measured by platelet survival tests. In addition *Carvalho et al* [4] and *Shattil et al* [21] have demonstrated that, in hyperbetalipoproteinemia platelets are *per se* hyperactive as a result of free cholesterol increase in the platelet membrane.

Our preliminary data suggest that platelet survival time in postmyocardial infarction patients without lipidic pattern abnormalities is comparable with the values in control subjects On the other hand, there was some interaction between lipemic abnormality and treatment in other words reduced survival time occurred in the hyperlipemic group treated with placebo but not in the group treated with sulfinpyrazone According to the data in literature, this efficacy of sulfinpyrazone does not seem to be correlated with any modification of platelet function investigated *ex vivo* and while sulfinpyrazone s efficacy in inhibiting platelet function *in vitro* has been known for some time [13 18 31] activity tests performed on platelets of subjects treated with the drug showed little or no alteration

As we have already suggested [6] the explanation of this phenomenon must lie in the fact that in the studies published [9] the agents inducing platelet aggregation were used at maximal doses. The *ex vivo* controlled findings on platelet bound ^{14}C-serotonin release reported here have evidenced that sulfinpyrazone has a significant inhibitory effect, which increases proportionately as the collagen concentration decreases.

Our results already reported in May 1977 [6] agree with those reported independently by *Ali and McDonald* [1] Furthermore, our recent findings show platelet aggregation inhibition in subjects treated with sulfinpyrazone if low concentrations of collagen and ADP are used [7] These data can be explained by the fact that sulfinpyrazone inhibits the synthesis of platelet prostaglandins and the aggregating intermediate endoperoxides competitively with the concentration of arachidonic acid and consequently with collagen concentration [1]

References

1 Ali, M and McDonald, J W D Effect of sulfinpyrazone on platelet prostaglandin synthesis and platelet release of serotonin. J Lab. clin. Med. 89 868–875 (1977).

2 Armitage P Statistical methods in medical research, pp 26_–268 (Blackwell Scientific Publications, Oxford 1971)

3 Brodsky I Siegel, N H Kahn, S. B. Ross, E. M and Petkov G Simultaneous fibrinogen and platelet survival with ^{75}Se-selenomethionine in man. Br J Haemat 18 341–355 (1970)

4 Carvalho, A. C. A. Colman, R. W and Lees, R. S. Pl telet functions in hyperbetalipoproteinemia. New Engl J Med. 290 434–438 (1974)

5 Cochran, W G and Cox, G M Experimental designs, p. 298 (Wiley New York 1964).

6 Cortellaro, M. Fassio, G de Ambroggi, L., and Polli E. E. Sulfinpyrazone in coronary artery disease (CAD) The Anturan Reinfarc

Acta haemat. 61: 75-79 (1979)

Platelet Dysfunction in Patients with Vitamin B_{12} Deficiency[1]

Steen Ingeberg and Erik Stoffersen

Section of Haematology, Department of Medicine, and Section of Blood Coagulation, Department of Clinical Chemistry, Aalborg Hospital, Aalborg

Key Words. Platelet · Platelet test · Pernicious anaemia

Abstract. 10 cases of pernicious anaemia are reported in which 6 had abnormal aggregation with epinephrine, 3 with fibrinogen, 2 with ADP and 2 with ristocetin. 5 patients had thrombocytopenia and 3 of these had a prolongation of the bleeding time. These abnormalities were normalized after vitamin B treatment. After treatment, platelet size changed toward a smaller diameter but platelet size, however, was not significantly different from the normal platelet size distribution.

Introduction

Thrombocytopenia is a common finding in patients with pernicious anaemia (PA). However, few studies have dealt with the platelet function in these patients. Abnormal platelet aggregation and decreased contents of lipids and phospholipids in the platelets have been demonstrated in some patients with PA. Therapy with vitamin B_{12} corrects the impaired platelet function [5, 10]. We have studied platelet size, platelet aggregation and the bleeding time in 10 patients with PA to elucidate how often platelet dysfunction may occur in this disease.

This work was supported by a grant from the Scientific Fund of the City of Aalborg.

Materials and Methods

The material comprises 10 unselected patients, 5 women and 5 men aged from 47 to 76 years (mean 68 years) with PA. The diagnosis PA was based on the following criteria: presence of megaloblasts in the bone marrow and macrocytes in the peripheral blood, on achlorhydria, even after maximal histamine stimulation, on the normalization of the erythropoietic bone marrow cells after vitamin B_{12} therapy and on positive Schilling test.

All patients were studied three times and some four times after the following schedule: before the Schilling test, 1 week after the Schilling test, and 4 weeks after initiation of vitamin B treatment. Furthermore patients with platelet function, not normalized after 4 weeks of therapy, were studied from 2 to 8 months after initiation of treatment.

Venous blood was drawn into plastic tubes after minimal stasis, and mixed (9+1) with 3.13 w/v trisodium citrate. All patients were instructed to

30 Zahavi, J Hyperaggregability of platelets in thromboembolic disorders. Thrombos. Haemost. *36* 479–482 (1976)
31 Zucker M B and Peterson, J.. Effect of acetylsalicylic acid, other nonsteroidal anti-inflammatory agents, and dipyridamole on human blood platelets. J Lab clin. Med. *76* 65–75 (1970)

Received February 6, 1978
Accepted. July 10 1978

Dr M. Cortellaro, Istituto di Clinica Medica I, Università di Milano, Via F Sforza 35,
I-20122 Milano (Italy)

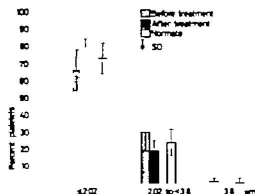


Fig. 1. Platelet size in 10 patients with PA before and after vitamin B treatment as compared to the results obtained from 26 normal persons

tients the platelet counts were above normal values. From table I it is obvious that a very low concentration of vitamin B_{12} is not necessarily associated with thrombocytopenia. Following vitamin B_{12} treatment platelet counts were normalized in all patients, and in 3 patients a transient thrombocytosis was seen.

Table II. Results of bleeding time (Ivy's method)

	I	II	III	IV
Normal range	6–12 min			
Patient				
1	13	10	11	4.5
2	18	7.5		4
3	11	9.5	6	6
4	15	6	6	5
5	4	3.5	3.5	3.5
6	5	5	5	
7	6	4	4.5	
8	6	4	4	
9	5.5	3.5	3.5	
10	6	5	5	

I = Before vitamin B_{12} therapy II–IV = after vitamin B_{12} therapy

There was no significant difference in platelet size both before and after vitamin B treatment compared to normals ($p > 0.05$ Student's t test) (fig. 1), but the patients showed a significantly greater number of smaller platelets after vitamin B_{12} treatment than before ($p < 0.05$ paired t test).

Table III. Results of platelet aggregation studies the aggregation curves were visually estimated as normal (N) or abnormal (A), compared to curves from 26 normal persons 4 patients with totally normal aggregation curves were excluded from the table

Patient		Aggregation agents				
		ADP	collagen	ristocetin	fibrinogen	epinephrine
1	I	A	–	A	A	A
	II	N		A	A	A
	III	N	N	A	A	A
	IV	N	N	N	N	A
2	I	N	N	N	N	A
	II	N	N	N	N	N
	III	N	N	N	N	N
	IV					
3	I	A		A	A	A
	II	N		A	N	A
	III	N		N	N	A
	IV	N	N	N	N	N
4	I	N	N	N	A	A
	II	N	N	N	A	A
	III	N	N	N	A	A
	IV	N	N	N	N	A
9	I	N	N	N	N	A
	II	N	N	N	N	N
	III	N	N	N	N	A
5	IV					
	I	N		N	N	A
	II	N	N	N	N	N
	III	N	N	N	N	N
	IV					

avoid any medication with a known effect on platelet function for a 10-day period prior to the investigation.

Platelet rich (PRP) and platelet poor plasma (PPP) were obtained by centrifugation of the blood immediately after sampling at room temperature for 4 min at 160 g and for 10 min at 1,500 g respectively. When necessary the platelet count of PRP was adjusted to a final count of 140,000–340,000 platelets/μl, by either recentrifugation or by addition of PPP.

Platelet aggregation *in vitro* was studied turbidimetrically according to the principles described by *Born* [3]. Evaluation of platelet aggregation was obtained with use of a Fibromat aggregometer (*Ble & Berntsen, Copenhagen, Denmark*). Within 2 h after sampling, PRP (300 μl) was placed in the plastic cuvette and stirred with a speed of 800 rpm at 37 °C. The aggregation was followed by continuous recording of light transmission as a function of time. The blank for each study was a similarly treated sample of PPP.

Aggregating agents used were: (a) adenosine 5 diphosphate (ADP: Sigma Chemical, Saint Louis, Mo.) 30 μl added to PRP to give a final concentration of 4.3 mol/l; (b) ristocetin sulphate (Lundbech & Co., Copenhagen, Denmark), 30 μl added to PRP to give a final concentration of 1.55 mg/ml; (c) epinephrine (The Hospital Pharmacy) 70 μl added to PRP to give a final concentration of 0.09 mg/ml; (d) bovine fibrinogen (Warner Chilcott), 20 μl added to PRP to give a final concentration of 0.27 mg/ml; (e) collagen (Mascia Brunelli, Milano, Italy) 20 μl added to PRP to give a final concentration of 18 μg/ml.

All aggregation curves were judged visually comparing with curves obtained from 26 healthy volunteers. Platelet size was measured according to the method of *Rivard and Lazerson* [8], modified by counting only 100 platelets. Bleeding time was measured by Ivy's method modified according to *Borchgrevink and Waaler* [2]. Platelet counts were determined using a *Thrombo-Counter* (Coulter Electronic). Serum vitamin B_{12} was assayed by the radioisotope dilution method [4].

Results

The results of haematologic tests of the 10 patients before and after treatment are summarized in table I. 5 of the 10 patients initially had thrombocytopenia, but in 2 pa-

Table I. Haematological values for 10 patients with PA

	Before treatment with vitamin B_{12}			After treatment with vitamin B_{12}		
	haemoglobin mmol/l	platelet count 10^9/l	vitamin B_{12} pmol/l	haemoglobin mmol/l	platelet count × 10^9/l	vitamin B_{12} pmol/l
Normal range	7.4–9.4 ♂ 7.6–10.6	140–340	220–660	7.4–9.4 7.6–10.6	140–340	220–760
Patient						
1♀	2.7	45	81	8.8	163	699
2♀	4.[illegible]	30	87	7.0	338	998
3	5.8	135	134	7.4	183	1,018
4	6.1	61	93	8.3	282	1,698
5♀	7.4	485	195	8.0	337	640
6♂	4.3	362	34	6.7	[illegible]54	723
7♂	4.8	19	137	8.9	240	2,745
8♂	6.9	78	86	8.3	219	736
9♂	7.3	238	103	8.5	251	1,787
10♂	9.2	769	12[illegible]	9.5	25[illegible]	3,000

gregation with ADP epinephrine and collagen, decreased PF 3 and a prolongation of the bleeding time. The defects were corrected with vitamin B_{12} therapy In the present series, 6 of 10 patients had impaired platelet aggregation. There was no change in the ristocetin aggregation after addition of normal plasma. Acquired abnormalities in ristocetin-induced aggregation have been observed in patients with infectious mononucleosis, idiopathic thrombocytopenia, and acute leukaemia. In these situations, defects in platelet aggregation to collagen or ADP are usually also present, as was found in our series. The defect is believed to involve the platelet intrinsically since it is not corrected by addition of normal plasma.

Thus, our findings confirm that besides the well-known abnormalities of platelet number abnormal platelet aggregation is present in many patients with PA. This thrombocytopathy is probably due to a defective megakaryocytopoiesis.

References

1 Araeth, J Über das qualitative (im engeren Sinne biologische) Verhalten der Blutplättchen bei der Perniciosa (qualitatives Thrombocytenblutbild) 30 Beobachtungen. Folia haemat., Lpz. 57 1–24 (1937)

2 Borchgrevink, C. F and Waaler B. A.. The secondary bleeding time. A new method for differentiation of hemorrhagic diseases. Acta med. scand. *162* 361 374 (1958).

3 Born, G V R.. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature, Lond. *194* 927–929 (1962).

4 Karstoft, H.. Serum vitamin B-12 bestemmelse med en isotopmetode (Universitetsforlaget, Aarhus 1971).

5 Levine, P H.. A qualitative platelet defect in severe vitamin B-12 deficiency Am. intern. Med. *78*. 533–539 (1969).

6 Ogston, D and Dawson, A. A.. Thrombocytosis following thrombocytopenia in man. Postgrad. med. J *45* 754–756 (1969).

7 Paddock, K. F and Smith, K. E.. The platelets in pernicious anemia. Am. J med. Sci. *198* 372 376 (1939).

8 Rivard, G. E. and Lazerson, J A simple method for platelet sizing. J Lab. clin. Med. *86* 547 550 (1975).

9 Smith, M D Smith, D A., and Fletcher M.. Haemorrhage associated with thrombocytopenia in megaloblastic anemia. Br med. J *i*. 982–985 (1962).

10 Stefanini, M and Karaca, M.. Acquired thrombocytopathy in patients with pernicious anaemia Lancet *i* 400–402 (1966).

Received: March 15, 1978
Accepted: May 25, 1978

Steen Ingeberg, Section of Haematology
Department of Medicine, Aalborg Hospital,
DK-9000 Aalborg (Denmark)

The bleeding time (table II) was prolonged in 3 cases, all had thrombocytopenia but none of the patients had any bleeding tendency. All patients had normal bleeding time after vitamin B_{12} therapy.

6 of the 10 patients (60%) showed abnormalities in the platelet aggregation (table III). The platelet aggregation curves were considered abnormal in cases of total absent aggregation, a maximal aggregation response less than in normals or no secondary aggregation phase. 2 patients had abnormal ADP induced aggregation before treatment, but 1 week after the Schilling test the ADP aggregation was normal. There was abnormal platelet aggregation induced by ristocetin, fibrinogen and epinephrine in 2, 3 and 6 patients respectively. These abnormalities were not normalized until 4 weeks of treatment with vitamin B_{12}. 3 patients still had impaired epinephrine aggregation which persisted after 8 months of vitamin B_{12} therapy. Slight impairment in epinephrine induced platelet aggregation is however observed with the same frequency in normals. The collagen induced aggregation was normal in all patients.

Discussion

The basic defect in the bone marrow of patients with vitamin B_{12} deficiency appears to be inability to synthesize DNA in the blood cell precursors in amounts adequate to allow orderly cell division. This affects all cell lines and eventually leads to the development of erythrocytopenia, granulocytopenia and thrombocytopenia.

Following therapy with vitamin B_{12} in some patients, a transient thrombocytosis may occur [6]. In the present series, 3 of 10 patients had elevated platelet counts 1 week after treatment with vitamin B_{12} was instituted. It has been proposed that the transient thrombocytosis may lead to thromboembolic complications. Thus, in each of two materials of 9 and 21 patients with PA, four thrombotic episodes occurred following therapy with vitamin B_{12} [5, 9]. In our material, 1 patient with thrombocytosis developed a deep venous thrombosis in the left leg.

6 of the patients had disturbed platelet function and only 3 of these patients had a prolonged bleeding time. Furthermore, there is no specific pattern of platelet dysfunction leading to a prolonged bleeding time. So there is no correlation between these findings. Concerning patient No. 8, it is our experience that the bleeding time is first abnormal with a platelet count below 50–60 × 10^9/l.

Platelet morphology in PA has received little attention. *Arneth* [1] described that bizarre forms including giant platelets may be found. A predominance of small platelets with occasional giant and other abnormal forms of platelet have been reported [7]. In the present study the platelets had a normal form and morphology. The platelet size measured before and after vitamin B_{12} therapy was not significantly different from the controls, but there was a significantly greater number of small platelets after vitamin B_{12} treatment than before.

Recently, qualitative platelet defects have been described in PA. Thus *Stefanini and Karaca* [10] found abnormal bleeding time and thromboplastin generation in 3 of 14 examined patients. Furthermore, there was a decreased content of lipids and phospholipids in the platelets. In 3 patients with PA, *Levine* [5] found impaired platelet ag-

gregation with ADP epinephrine and collagen, decreased PF 3 and a prolongation of the bleeding time. The defects were corrected with vitamin B_{12} therapy In the present series, 6 of 10 patients had impaired platelet aggregation. There was no change in the ristocetin aggregation after addition of normal plasma. Acquired abnormalities in ristocetin-induced aggregation have been observed in patients with infectious mononucleosis, idiopathic thrombocytopenia, and acute leukaemia. In these situations, defects in platelet aggregation to collagen or ADP are usually also present, as was found in our series. The defect is believed to involve the platelet intrinsically since it is not corrected by addition of normal plasma.

Thus, our findings confirm that besides the well-known abnormalities of platelet number abnormal platelet aggregation is present in many patients with PA. This thrombocytopathy is probably due to a defective megakaryocytopoiesis.

References

1 Arneth, J Über das qualitative (im engeren Sinne biologische) Verhalten der Blutplättchen bei der Perniciosa (qualitatives Thrombocytenblutbild). 30 Beobachtungen. Folia haemat., Lpz. 57 1 24 (1937).

2 Borchgrevink, C. F and Waaler B. A. The secondary bleeding time. A new method for differentiation of hemorrhagic diseases Acta med. scand. 162 361–374 (1958).

3 Born, G V R Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature, Lond. 194 927–929 (1962).

4 Karnoft, H. Serum vitamin B-12 bestemmelse med en isotopmetode (Universitetsforlaget, Aarhus 1971).

5 Levine, P H. A qualitative platelet defect in severe vitamin B-12 deficiency Ann intern Med. 78. 533–539 (1969)

6 Ogston, D and Dawson, A. A. Thrombocytosis following thrombocytopenia in man. Post grad. med J 45 754–756 (1969).

7 Paddock, K. F and Smith, K. E. The platelets in pernicious anaemia Am. J med. Sci. 198. 372 376 (1939).

8 Rivard, G E. and Lazerson, J A simple method for platelet aggreg. J Lab clin. Med. 86. 547 550 (1975).

9 Smith, M. D., Smith, D A and Fletcher M Haemorrhage associated with thrombocytopenia in megaloblastic anaemia. Br med J i. 982–985 (1962).

10 Stefanini, M. and Karaca, M. Acquired thrombocytopathy in patients with pernicious anaemia Lancet i. 400–402 (1966).

Received: March 15 1978
Accepted. May 23, 1978

Steen Ingeberg, Section of Haematology
Department of Medicine, Aalborg Hospital,
DK-9000 Aalborg (Denmark)

The bleeding time (table II) was prolonged in 3 cases, all had thrombocytopenia, but none of the patients had any bleeding tendency. All patients had normal bleeding time after vitamin B_{12} therapy.

6 of the 10 patients (60%) showed abnormalities in the platelet aggregation (table III). The platelet aggregation curves were considered abnormal in cases of total absent aggregation, a maximal aggregation response less than in normals or no secondary aggregation phase. 2 patients had abnormal ADP-induced aggregation before treatment but 1 week after the Schilling test the ADP aggregation was normal. There was abnormal platelet aggregation induced by ristocetin, fibrinogen and epinephrine in 2, 3 and 6 patients, respectively. These abnormalities were not normalized until 4 weeks of treatment with vitamin B_{12}. 3 patients still had impaired epinephrine aggregation which persisted after 8 months of vitamin B_{12} therapy. Slight impairment in epinephrine induced platelet aggregation is, however, observed with the same frequency in normals. The collagen-induced aggregation was normal in all patients.

Discussion

The basic defect in the bone marrow of patients with vitamin B_{12} deficiency appears to be inability to synthesize DNA in the blood cell precursors in amounts adequate to allow orderly cell division. This affects all cell lines and eventually leads to the development of erythrocytopenia, granulocytopenia and thrombocytopenia.

Following therapy with vitamin B_{12}, in some patients, a transient thrombocytosis may occur [6]. In the present series, 3 of 10 patients had elevated platelet counts 1 week after treatment with vitamin B_{12} was instituted. It has been proposed that the transient thrombocytosis may lead to thromboembolic complications. Thus, in each of two materials of 9 and 21 patients with PA, four thrombotic episodes occurred following therapy with vitamin B_{12} [5, 9]. In our material 1 patient with thrombocytosis developed a deep venous thrombosis in the left leg.

6 of the patients had disturbed platelet function and only 3 of these patients had a prolonged bleeding time. Furthermore there is no specific pattern of platelet dysfunction leading to a prolonged bleeding time. So there is no correlation between these findings. Concerning patient No. 8, it is our experience that the bleeding time is first abnormal with a platelet count below 50–$60 \times 10^9/l$.

Platelet morphology in PA has received little attention. *Arneth* [1] described that bizarre forms including giant platelets may be found. A predominance of small platelets with occasional giant and other abnormal forms of platelet have been reported [7]. In the present study the platelets had a normal form and morphology. The platelet size measured before and after vitamin B_{12} therapy was not significantly different from the controls, but there was a significantly greater number of small platelets after vitamin B_{12} treatment than before.

Recently, qualitative platelet defects have been described in PA. Thus, *Stefanini and Karaca* [10] found abnormal bleeding time and thromboplastin generation in 3 of 14 examined patients. Furthermore, there was a decreased content of lipids and phospholipids in the platelets. In 3 patients with PA, *Levine* [5] found impaired platelet ag

shared one haplotype as demonstrated in figure 1 It should be mentioned that both parents were of Russian-Jewish ancestry however there was no possibility to confirm or rule out consanguinity The haplotype AW26-BW38 seems to be rather frequently found in this population [*G Opelz*, pers. commun]. A mixed lymphocyte culture (MLC) was performed on two occasions [5] and it was found that the patient and her father were also MLC nonreactive indicating identity for the HLA-D locus The results of the two MLC experiments are summarized in table I. The patient was considered to be resistant to conventional therapy when she was referred to the City of Hope National Medical Center for BMT at this time she was in her fifth relapse.

Clinical Course

Because donor and recipient showed major blood group incompatibility (see fig. 1) total plasma exchange using continuous flow cell separator was done prior to transplantation on day 1 without technical difficulties. The anti-A titer which was positive 1:32 at the beginning was negative after the procedure and throughout the course (see table II) Conditioning measures included administration of 60 mg of cyclophosphamide/kg body weight on two successive nights (day -4 and 3) and total body irradiation with 1,000 rad midline tissue dose was performed on day 0. Thereafter bone marrow was obtained from the patient's father by multipl needle aspirations [9] and infused into the patient (6.8×10^8 nucleated cells/kg body weight) through right atrial catheter [2]. After BMT methotrexate was given for prevention/amelioration of graft-versus-host disease (GVHD) following widely accepted schedule [8, 10]

After transplantation the whit blood cell count dropped to range of 18–98/ l for approximately 10 days On day 18 the white blood cells had risen to 1,000/µl and returned to normal during the weeks thereafter During this phase of

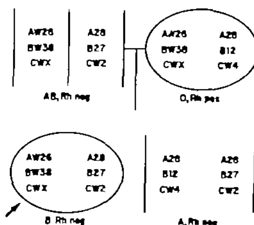


Fig. 1. HLA genotypes of blood groups of the patient (arrow) and her family

Table I. Mixed lymphocyte culture between donor recipient, and unrelated controls

Responding cell	Exp. No	Stimulating cell (irradiated)								
		recipient (daughter)			donor (father)			control (unrelated)		
		CPM	SD %	SR	CPM	SD %	SR	CPM	SD %	SR
Recipient	1	188	5	1 0	225	29	1.2	4,774	34	25 4
(daughter)	2	187	48	1.0	199	52	1 1	4,626	13	30 1
Donor	1	327	30	_1	156	39	1 0	4,228	11	27 1
(father)	2	493	31	0 8	661	17	1 0	9 712	5	14 7
Control	1	3,797	12	9.0	3,376	8	8 0	424	45	1.0
(unrelated)		4,18_	11	8 5	5,357	15	10.9	491	16	1 0

Acta haemat. 61 80–84 (1979)

Bone Marrow Transplantation for Acute Leukemia Using a Histocompatible Paternal Donor[1]

K G Blume K J Bross R K Chillar D O Findley J H Fuerth R R. Palodugu D Sharkoff and J A Warner

City of Hope National Medical Center Duarte, and Kaiser Permanente Medical Center Panorama City Calif

Key Words. Acute leukemia Bone marrow transplantation Graft versus host disease Terminal deoxynucleotidyl transferase

Abstract. A bone marrow transplantation from an HLA identical MLC nonreactive paternal donor has been performed in a patient with acute lymphoblastic leukemia resistant to drug treatment. Prompt engraftment was documented however the patient died of interstitial pneumonitis due to cytomegalovirus 65 days after transplant. Clinical manifestation of graft versus host reaction was mild. Recurrence or persistence of leukemia was found at the time of death using cytogenetic markers and determination of the leukemic marker enzyme terminal deoxynucleotidyl transferase

Introduction

Bone marrow transplantation (BMT) between histocompatible siblings has become a rather frequently practiced procedure in the treatment of acute leukemia and aplastic anemia during recent years [8 10] Occasionally incompatible paternal bone marrow has been transplanted however these attempts were not successful [4] Recently a successful marrow transplantation has been reported in which a histocompatible maternal donor was used [3] Although the follow up time in this case is still short, the result seems to be encouraging. We report herein another case in which a patient with acute leukemia, resistant to conventional therapy received a bone marrow transplant from her histocompatible father

[1] This work was made possible in part by support from the Thomas J Thorley Research Fund

Case History

The patient was 5 years old when acute lymphoblastic leukemia was diagnosed in April 1975 A complete remission was induced which lasted about 1 year thereafter the patient had several leukemic relapses including manifestation of CNS involvement. HLA typing [7] for the HLA loci A, B, and C showed that the patient and her father were HLA identical and that both parents

percellular marrow and an increase in the number of immature myelocytic elements. Cytogenetic analysis of the postmortem bone marrow sample showed the presence of a small proportion of female bone marrow cells and the activity of TDT was above normal limits, both indicating a recurrence of leukemia (see table II). Histological examination of the skin and gastrointestinal tract showed no signs of GVHD but in the liver grade II GVHD was found.

Discussion

A BMT has been performed in an end-stage leukemia patient from her HLA-A, B and C identical MLC nonreactive father. A plasma exchange to correct for a major blood group mismatch was carried out prior to transplantation without technical difficulties. Engraftment was confirmed by clinical and cytogenetic parameters and the patient survived the initial septic period of several weeks and was clinically stable thereafter. However 2 months after transplantation interstitial pneumonitis due to CMV occurred and the patient died of respiratory failure. Evaluation of a postmortem bone marrow specimen indicated a recurrence of leukemia with an increase in number of immature myelocytic elements, some female bone marrow cells on cytogenetic analysis, and an increase in activity of the leukemic marker enzyme TDT. It is not possible to decide whether the recurrence of leukemia had occurred in donor cells, however it is more likely that the leukemic relapse was due to persistence of leukemia resistant to the conditioning regimen administered. Clinical and histological manifestation of GVHD was not striking.

References

1 Beutler, E. and Kuhl, W.: The assay of leukocyte and bone marrow terminal deoxynucleotidyl transferase. Am. J. clin. Path. (in press, 1978).

2 Broviac, J. W.; Cole, J. J. and Scribner, B. H.: A silicone rubber atrial catheter for prolonged parenteral alimentation. Surgery Gynec. Obstet. 136: 602–606 (1973).

3 Falk, P. M.; Feig, S. A., and Gale, R. P.: Successful bone marrow transplantation for acute leukemia using histocompatible maternal donor. Expl. Hematol. 5: suppl. 2, p. 102 (1977).

4 Feig, S. A.; Falk, P. M.; Neerhout, R. C.; Sparkes, R.; Gale, R. P.; Opelz, G.; Cline, M. J.; Fahey, J. L.; Smith, G.; Saran, G.; Territo, M.; Young, L.; Langdon, E. A., and Fawzi, F.: Experience with incompatible maternal donors for bone marrow transplantation. Blut 34: 1–20 (1977).

5 Hartzman, R. J.; Segall, M.; Bach, M. L., and Bach, F. H.: Histocompatibility matching. VI. Miniaturization of the mixed leukocyte test. A preliminary report. Transplantation 11: 268–273 (1971).

6 Lerner, K. G.; Kao, G. R.; Storb, R.; Buckner, C. D.; Clift, R. A., and Thomas, E. D.: Histopathology of graft-vs-host reaction (G HR) in human recipients of bone marrow from HL-A matched sibling donors. Transplantn Proc. 6: 367–371 (1974).

7 Mittal, K. K.; Mickey, M. R.; Singal, D. P., and Terasaki, P. L.: Refinement of microdroplet lymphocyte cytotoxicity test. Transplantation 6: 913–927 (1968).

8 Storb, R.; Thomas, E. D.; Weiden, P. L.; Buckner, C. D.; Clift, R. A.; Fefer, A.; Fernando, L. P.; Giblett, E. R.; Goodell, B. W.; Johnson, F. L.; Lerner, K. G.; Neiman, P. E., and Sanders, J. E.: Aplastic anemia treated by allogeneic bone marrow transplantation. A report on 49 new cases from Seattle. Blood 48: 817–841 (1976).

9 Thomas, E. D. and Storb, R.: Technique for human marrow grafting. Blood 36: 507–515 (1970).

pronounced leukopenia daily granulocyte support from the bone marrow donor and a partly HLA compatible unrelated blood donor was given. The patient's platelet count also dropped markedly and platelet transfusions were necessary intermittently Packed red blood cells were also transfused repeatedly to correct for pronounced anemia. Cytogenetic studies repeatedly revealed the presence of donor-type bone marrow cells confirming engraftment as demonstrated in table II Moreover bone marrow cellularity returned to normal with all three cell lines represented. Determination of bone marrow terminal deoxynucleotidyl (TDT) [1] showed reduction to normal activity of this leukemic marker enzyme.

The patient had a septic course during the first month and received antibiotic drugs in combination (gentamicin, cephalothin and carbenicillin) as well as amphotericin B Prednisone was given to decrease systemic reactions related to amphotericin B. Further supportive therapy consisted of parenteral hyperalimentation with glucose-amino acid solutions. The course was further complicated by severe hemorrhagic, herpetic mucositis and intermittent cardiac failure, probably due to cyclophosphamide

On day 25 a maculopapular skin rash appeared which was consistent with clinical grade II GVHD A skin biopsy confirmed this finding [6]. The rash disappeared after about 3-5 days. Throughout the whole course liver function studies remained largely within normal limits except for a temporary rise of SGOT and SGPT to 150 and 183 U respectively and there was only mild intermittent diarrhea indicating low grade GVHD involving the gastrointestinal tract.

After about 2 months another rise of temperature occurred. This was accompanied with shortness of breath and dry intractable cough. At this time a chest X ray revealed typical signs of bilateral interstitial pneumonia and blood gas analysis showed decreasing arterial oxygen tension. The patient required mechanical ventilation under muscle relaxation but the respiratory situation worsened and the patient expired 65 days after transplantation.

At autopsy extensive bilateral interstitial pneumonia due to cytomegalovirus (CMV) and hyaline membrane disease was found. Sections of the bone marrow revealed a hy

Table II. Cytogenetic findings, terminal deoxynucleotidyl transferase determinations and anti-A titer prior to transplantation and during the posttransplantation course

	Bone marrow karyotype XX	XY	not evaluable	terminal deoxynucleotidyl transferase (normal < 19 8 µU/mg protein)	Plasma anti-A titer
Pretransplant day − 6	100%	0	0	799 3	1 32
Posttransplant day + 11	0	46	4	0.0	neg.
Posttransplant day + 25	0	29	1	0 0	neg.
Posttransplant day + 40	0	22	4	8 9	neg.
Posttransplant day + 54	1 (?)	47	2	0 0	neg.
Posttransplant day + 65	2	20	3	80 4	

1 Karyotypes from lymphocyte and fibroblast cultures
Postmortem sample.

Acta haemat. 61 85–92 (1979)

Diagnostic Value of Leucocytic Acid-Phosphatase Isoenzymes in Determining Cytological Types of Non Lymphoid Acute Leukaemias

S Woessner R Lafuente J Capdevila and J Sans Sabrafen

Department of Haematology nd Clinical Oncology Hospital de la Cruz Roja, Barcelona

Key Words. Acid phosphatase Isoenzymes Leukaemia, monoblastic Leukaemia, myeloblastic

Abstract. The normal isoenzymatic pattern of leucocytic acid-phosphatase based on the study of 150 haematologically normal individuals is reported. The different pathologic patterns of the leucocytic acid-phosphatase isoenzymes occurring in non-lymphoblastic *acute leukaemias are presented and correlated with the subdivisions of acute leukaemias* established by the French-American-British (FAB) Co-operative Group. This study is considered to be especially useful in identifying pure acute monocytic leukaemias corresponding to subtype M of the FAB as well as acute erythraemias with unusual cytological and cytochemical features.

The usefulness of cytochemistry in the filiation of blastic cells is a well known and universally accepted fact. Although certain morphological data such as the presence of Auer bodies and of dysgranulopoietic features are definite or highly suggestive indications of acute myelocytic leukaemia, in the majority of cases these morphological criteria should be complemented by cytochemical analysis. This concept is also supported by the French-American-British (FAB) Co-operative Group [2] which has recently established a series of subdivisions of acute leukaemias on the basis of morphological and cytochemical characteristics. According to the degree of cell maturity and the differentiation into one or more cell lines, three lymphoblastic (L_1 L_2, L_3) and six myeloid (M M_2, M_3, M_4, M_5, M_6) varieties have been identified. Only the myeloid subtypes were included in our study The first three types (M M_2, M_3) exhibit predominant granulocytic differentiation, the fourth (M_4) mixed granulocytic and monocytic, and the fifth (M_5) monocytic exclusively Erythroblastic and granulocytic differentiation coexist in the M_6 subvariety

We should emphasize that the detection of myeloperoxidase at the optical or ultrastructural level is conclusive evidence for a myeloblastic filiation. Similarly an abundance of fluoride-sensitive, non-specific es-

10 Thomas, E. D. Buckner, C. D., Banaji, M., Clift, R. A. Fefer, A. Flournoy, N. Goodell, B. W. Hickman, R. O. Lerner, K. G. Neiman, P. E. Sale, G. E. Sanders, J. E. Singer, J. Stevens, M. Storb, R., and Weiden, P. L.: One hundred patients with acute leukemia treated by chemotherapy, total body irradiation, and allogeneic marrow transplantation. Blood 49: 511–533 (1977).

Received: April 13, 1978
Accepted: July 14, 1978

K. G. Blume, MD
Department of Bone Marrow Transplantation,
City of Hope National Medical Center
1500 East Duarte Road, Duarte, CA 91010
(USA)

migration rates when using α-naphthyl phosphate substrate were observed. They were identified with bands 2, 3 and 4 obtained by *Li et al* [7] on polyacrylamide gel (fig. 1) Isoenzymatic band 2 has a monocytic and granulocytic origin, while bands 3 and 4 exhibit, respectively lymphocytic and *a predominant monocytic origin. Incubation* using naphthol-As-Bi-phosphate substrate, which is specific for lymphoid cells, hydrolyzed lymphocytic band 3 in preference to monocytic band 4 thus, an evident band 3 and a weak band 4 constituted the isoenzymatic pattern (fig. 2)

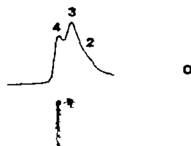
The relative percentages of each band were obtained from the densimetric reading of the electrophoretic diagram with α-naphthyl-phosphate substrate. Mean values and standard deviations are shown in table II

Table II. Leucocytic acid-phosphatase isoenzymes in 150 normal controls

Isoenzymatic bands α-naphthyl-phosphate substrate	Relative percentages $\bar{x}$	SD
Band 2	25 11	5.89
Band 3	44 4	4 9
Band 4	30 9	5.9



Fig. 1. Normal isoenzymatic pattern α-naphthyl-phosphate substrate



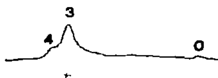


Fig. 2. Normal isoenzymatic pattern, naphthol-As-Bi-phosphate substrate.

4 2 O

Fig. 3. Normal control (top) and control inhibited with L(+)-tartaric acid α-naphthyl-phosphate substrate

terases would indicate the monocytic origin of a particular cell Surprisingly the detection of acid hydrolases has been considered of limited value in classifying acute leukaemias Some authors for example have reported that cytochemical evidence of β-glucuronidase is of no diagnostic value at all [12] Others, however consider that a diffuse or granular type of positive reaction would characterize respectively myeloid and lymphoid leukaemias [9]

Cytochemical detection of acid phosphatase though of remarkable value in identifying proliferative plasmocytosis [3] erythraemias [10] Gaucher s cellularity [8] and hairy cells [8] is of limited use in determining non lymphoid acute leukaemias On the other hand a granular positivity of centrosomic localization in acute lymphoid leukaemias suggests their immunological T cell origin [4] As for the presence of acid phosphatase in non lymphoid acute leukaemias it is well known that it is found in greater abundance in the monoblasts than in the myeloblasts, and also that its enzymatic activity appears cytochemically as a diffuse precipitate Until recently however this did not provide any further help

Over the past year we have given special attention to combining cytochemical and isoenzymatic studies of the acid phosphatase Our aim was to achieve a more precise evaluation of chronic lymphoproliferative disorders with peripheral blood manifestations [6] When this methodology was applied to the study of acute leukaemias of unquestionable myeloid origin we found that the isoenzymatic patterns of leucocytic acid phosphatase were different for each of the subtypes of acute myeloid leukaemias studied by us and established in the FAB classification. We believe that the electrophoretic patterns of leucocytic acid phosphatase may be another useful parameter in distinguishing the subtypes of acute myeloid leukaemias

Materials and Methods

The isoenzymes of leucocytic acid phosphatase were studied in a series of 150 haematologically normal controls and in 10 patients with acute myeloid leukaemias, cytochemically confirmed. Table I shows the distribution of the cases according to the criteria of the FAB subclassification [2]

Isoenzymes of acid phosphatase were determined following the techniques of *Axline* [1] with personal modifications [6] cellulose acetate was used for medium instead of polyacrylamide gel. Two substrates, α-naphthyl-phosphate and naphthol As-Bi-phosphate, were used to detect the different isoenzymatic bands. In some cases the tartrate sensitivity or resistance of the isoenzymatic bands was tested by incubating them in L(+)-tartaric acid according to the technique proposed by *Yam et al* [14]

Results

In the 150 haematologically normal controls three isoenzymatic bands with different

Table I. Distribution of 10 patients with acute myeloid leukaemia

Morphological variant[1]	Number of cases
M	
M_2	1
M	0
M	4
M_5	3
M	0

FAB classification [2]

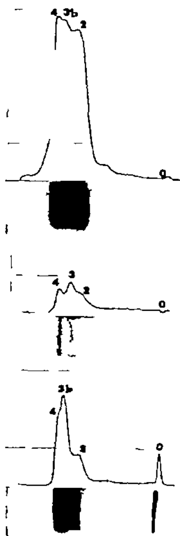


Fig. 6. Isoenzymatic pattern of an acute myeloid leukaemia type M (bands 2, 3b and 4): α-naphthyl-phosphate substrate (top) and naphthol-As-Bi-phosphate substrate (bottom); normal control in the middle.

subtype) determined an extremely intense band 4 regardless the degree of cell maturity; band 3 b was absent and band 2 appeared in a lesser proportion (fig 5)

Patients with acute myelomonocytic leukaemias (FAB M subtype) presented a supernumerary band 3b coexisting with a reinforced band 4 as was expected. The electrophoretic separation of the two bands was more easily visible with the substrate naphthol As-Bi-phosphate (fig. 6) In no ca of non-lymphoid acute leukaemia could t isoenzymatic band 5 be observed

The white blood cell counts and the t tive percentages of the different bands responding to each patient are referre table III

Discussion

The combination of conventional morphological, cytochemical and ultrastructural studies with the analysis of the cell markers has shown that in almost all cases the term 'non-differentiated' acute leukaemia should be abolished. Nevertheless, there still are some cases of uncertain filiation because such a complete methodology may not be practicable The alue of cytochemical analysis of myeloperoxidase in the filiation of a blastic cellularity is universally accepted. As for the cytochemical study of leucocytic *acid phosphatase*, a *faint and diffusely distributed* cytoplasmic positivity characterizes myeloblasts, while monoblasts exhibit a more intense positi ity This is a subjective quantitative evaluation, however and subject to various technical conditions. The isoenzymatic study of acid phosphatase is a more *objective methodology giving diverse* patterns for different non-lymphoid acute leukaemias. We consider that this simple exploratory technique could be applied in the routine haematologic methodology for the diagnosis of blastic populations.

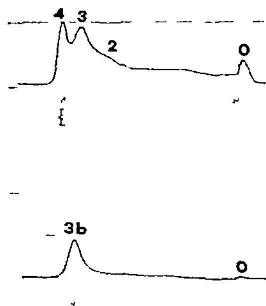


Fig. 4 Isoenzymatic pattern of an acute myeloid leukaemia type M (band 3b) normal control at the top α-naphthyl-phosphate substrate.

The inhibition of acid phosphatase activity with L(+)-tartaric acid revealed a partial resistance of bands 2 and 4 and a total inhibition of bands 3 and 3b (fig 3)

Leucocytic isoenzymatic patterns of acid phosphatase in patients with acute myeloid leukaemias differed according to their morphological types In the M_1 and M subtypes a supernumerary band, labeled 3b appeared when using both substrates. In cases of absolute blastosis it appeared alone, but when a few mature leucocytes remained it was accompanied by faint bands 2, 3 and 4 (fig 4)

The Schilling s type morphological variety of pure monocytic leukaemia (FAB M

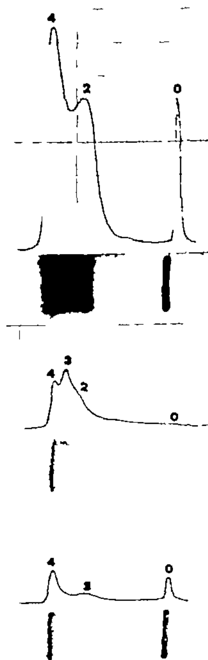


Fig. 5. Isoenzymatic pattern of an acute myeloid leukaemia type M (increase of bands 2 and 4) α-naphthyl-phosphate substrate (top) and naphthol As-BI-phosphate substrate (bottom) normal control in the middle.

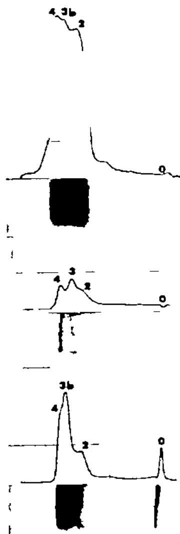


Fig. 6. Isoenzymatic pattern of an acute myeloid leukaemia type M (bands 2, 3b and 4); a-naphthyl-phosphate substrat (top) and naphthol-As-Bi-phosphate substrate (bottom); normal control in the middle.

subtype) determined an extremely intense band 4 regardless the degree of cell maturity: band 3 b was absent and band 2 appeared in a lesser proportion (fig. 5).

Patients with acute myelomonocytic leukaemias (FAB M subtype) presented a supernumerary band 3b coexisting with a reinforced band 4 as was expected. The electrophoretic separation of the two bands was more easily visible with the substrate naphthol-As-Bi-phosphate (fig. 6). In no case of non-lymphoid acute leukaemia could the isoenzymatic band 5 be observed.

The white blood cell counts and the relative percentages of the different bands corresponding to each patient are referred in table III

Discussion

The combination of conventional morphological, cytochemical and ultrastructural studies with the analysis of the cell markers has shown that in almost all cases the term 'non-differentiated' acute leukaemia should be abolished. Nevertheless, there still are some cases of uncertain filiation because such a complete methodology may not be practicable. The value of cytochemical analysis of myeloperoxidase in the filiation of a blastic cellularity is universally accepted. As for the cytochemical study of leucocytic acid phosphatase, a faint and diffusely distributed cytoplasmic positivity characterizes myeloblasts, while monoblasts exhibit a more intense positivity. This is a subjective quantitative evaluation, however and subject to various technical conditions. The isoenzymatic study of acid phosphatase is a more objective methodology giving diverse patterns for different non-lymphoid acute leukaemias. We consider that this simple exploratory technique could be applied in the routine haematologic methodology for the diagnosis of blastic populations.

Table III Study of leucocytic acid-phosphatase isoenzymes in 10 patients with acute myeloid leukaemia

Case	Cytological type (FAB)	Leucocytes × 10⁹/l	Differential count				Isoenzymatic study bands[1]				
			G	L	M	Mbl	2	3	3b	4	5
1	M_1	145	0	4	0	96	0	0	100	0	0
2	M_1	85	0	3	4	93	0	0	90	10	0
3	M_2	65	35	5	0	60	—	0	40	[illegible]	0
4	M_4	95	29	5	28[2]	38	26	0	34	40	0
5	M_4	250	1	2	31[2]	66	15	0	20	65	0
6	M_4	195	1	6	60[2]	34	25	0	35	40	0
7	M	135	1	2	67[2]	30	26	0	34	40	0
8	M_5	170	1	7	9[3]	0	8	0	0	77	0
9	M_5	180	7	7	86[3]	0	6	0	0	74	0
10	M	340	4	4	9[3]	0	6	0	0	74	0

G = Granulocytes L = lymphocytes M = monocytes Mbl = myeloblasts.

[1] Relative percentages of the isoenzymatic bands.

[2] Well-differentiated atypical monocytes.

[3] Undifferentiated monocytic variant (monoblasts).

In interpreting the electrophoretic diagram, special attention should be drawn to the proportions of the normal bands and to the eventual appearance of supernumerary ones. For example, the so-called band O is specific for the thesaurismotic Gaucher's cell [7] band 3b corresponds to blastic cellularity [7] and band 5 up to now has only been seen in a significant proportion in cases of hairy-cell leukaemia [7] The use of two different substrates helps in the correct identification of the bands, allowing clear visualization within a few millimeters. Furthermore the hydrolytic capacity of the bands with each substrate also constitutes a differential characteristic thus, naphthol As-Bi-phosphate is specific for bands 3 3b and 5 [7] We believe that the partial resistance to L(+)-tartaric acid of bands 2 and 4 is due to their monocytic component which would eventually confirm the findings of *Moyer et al* [11] they reported a partial resistance of the cells of the phagocytic mononuclear system in a quantitative study of the activity of tartrate resistant acid phosphatase. Band 3b proved to be totally tartrate sensitive in all of our cases, just as in the studies by *Li et al* [8] Of all the isoenzymatic bands of acid phosphatase detected with α-naphthyl phosphate substrate only band 5 occurring in hairy-cell leukaemia [14] showed a constant and total resistance to $L(+)$-tartaric acid.

The use of acid phosphatase isoenzymes is especially valuable in identifying the poor differentiated monoblastic variant (M_5) of pure monocytic leukaemias, whose diagnosis on the basis of morphological criteria alone is often impossible. We have not observed band 3b in cases of monocytic leukaemia, regardless of the degree of cellular differentiation on the other hand, a very intense band 4 which is indicative of monocytic cells, was noticed The absence of a supernumerary band 3b allows its differen-

tiation from the myeloblastic and lymphoblastic proliferations.

A supernumerary band 3b was present in the subtypes M_1 and M_2, which were not distinguished in this way from acute lymphoid leukaemias. This band was labeled 3b because of its origin in the blastic cellularity and its proximal situation to the normal band 3. A somewhat weaker band 3b coexisting with a slight proportion of the normal bands could be expected in the M_2 subtype, due to the existence of a certain degree of cell maturity and correspondingly a lower proportion of blastic infiltration.

Thus far we have not had the opportunity to study the isoenzymatic pattern of leucocytic acid phosphatase in the M_3 subtype or promyelocytic variant. It seems logical that we should not observe band 3b since this *leukaemic proliferation corresponds to* a maturative phase beyond the blastic stage. In the mixed myelo-monocytic variety (FAB M_4 subtype) a band 3b characteristic of the myeloblastosis and a strong band 4 reflecting the monocytic component coexist.

In the erythroleukaemia or M_6 variant, only band 3b would be expected and that in proportion to the degree of myeloblastosis. On the other hand, the blastosis of the erythrocytic precursors does not show the band 3b in the isoenzymatic pattern because of the peculiar physicochemical characteristics of the acid phosphatase in these blastic cells (low molecular weight and absence of hydrolysis with the substrates used in our technique) [5, 13]. We have recently observed a case of pure erythraemia without band 3b [13]. We feel that a comment on pure erythraemia is justified, even though it does not form part of the results under consideration, since in common practice a peripheral blastosis cytochemically positive for acid phosphatase without the appearance of band 3b or a reinforcement of band 4 in the isoenzymatic pattern using α-naphthyl-phosphate substrate strongly indicates the pure erythraemic nature of the cell proliferation.

Although this is only a preliminary study we believe it points toward the practical utility of leucocytic acid phosphatase isoenzymes to establish a more accurate classification of the subtypes of non-lymphoid acute leukaemias.

References

1 Axline, S. G.: Isoenzymes of acid-phosphatase in normal and Calmette-Guérin bacillus-induced rabbit alveolar macrophages. J. exp. Med. *128:* 1031–1048 (1968).

2 Bennet, J. M.; Catovsky, D.; Daniel, M. T.; Flandrin, G.; Galton, D. A. G.; Gralnick, H. R., and Sultan, C.: Proposals for the classification of acute leukaemias. French-American-British (FAB) Co-operative group. Br. J. Haemat. *33:* 451–458 (1976).

3 Canuto, J. P.; Hammon, J. C.; Pastorelli, E.; Dujardin, P., and Masseyeff, R.: Plasma cell acid phosphatase, a discriminatory test for benign and malignant monoclonal gammopathies. Biomedicine *5:* 197–199 (1977).

4 Catovsky, D.; French, B., and Van Noorden, S.: B, T and null cell leukaemias. Electron cytochemistry and surface morphology. Blood Cells *1:* 115–124 (1975).

5 Hollander, V. P.: Hydrolysis. Other C-N bonds: phosphate esters; in Boyer: The enzymes, vol. 4, pp. 449–498 (Academic Press, New York 1971).

6 Lafuente Rodes, R.: Estudio citoquímico isoenzimático leucocitario de las *fosfatasas ácidas* en los síndromes linfoproliferativos crónicos con expresión hemoperiférica; tesis Barcelona (1977).

7 Li, C. Y.; Yam, L. T., and Lam, K. W.: Acid-phosphatase isoenzymes in human leukocytes in normal and pathologic conditions. J. Histochem. Cytochem. *18:* 473–481 (1970).

8 Li, C. Y Yam, L. T., and Lam K. W Studies of acid phosphatase isoenzymes in human leukocytes. Demonstration of isoenzyme cell specificity J Histochem. Cytochem. *18* 901–910 (1970).
9 Lorbacher P Zur diagnostischen Anwendung des cytochemischen β glucuronidase-Nachweises, in Gross and Van de Loo Leukämie (Springer Berlin 1972)
10 Meister H und Fuchs, R. Zytochemie der malignen Erythroblastose. Folia haemat., Lz. *1* 71–80 (1972).
11 Mover S. Li C. Y and Yam, L. T., Semi quantitative evaluation of tartrate-resistant acid phosphatase activity in human blood cells. J Lab clin. Med *80* 711–717 (1972)
12 Shaw M T and Klemp, V A. Cytochemical staining with oil red 0 and for β glucuronidase in the diagnosis of acute leukaemia. Am. J clin. Path. *61* 169–172 (1974).
13 Woessner S. Lafuente Rodes, R., and Sans-Sabrafen, J. Valor del estudio combinado citoquímico e isoenzimático de la fosfatasa ácida en la identificación de la población blastica eritrémica Sangre, Barcelona (in press).
14 Yam, L. T Li, C. Y., and Lam, K. W., Tartrate-resistant acid phosphatase isoenzyme in the reticulum cells of leukemic reticuloendotheliosis. New Engl. J Med. *284* 357 360 (1971).

Received December 28, 1977
Accepted, April 22, 1978

S. Woessner MD
Department of Haematology
Hospital de la Cruz Roja,
Dos de Mayo 301 Barcelona (Spain)

Acta haemat. *61* 93–99 (1979)

A Long-Term Follow Up Study in Essential Cryoglobulinemia

F Invernizzi P Pioltelli R Cattaneo V Gavazzeni P Borzini, G Monti and C Zanussi

4th Medical Clinic and 4th Institut of Medical Pathology University of Milan, Milan, and Ente Ospedaliero Provinciale, Saronno

Key Words. Complement Cryoglobulinemia DNA antibodies Glomerulonephritis Hepatic cirrhosis Lymphoproliferative disease

Abstract. In a case series of 56 patients with essential cryoglobulinemia, 35 were followed-up for 4–13 years (mean 7 years) A membranous proliferative glomerulonephritis, which in about half the cases showed a progression to renal insufficiency was the commonest complication, observed in more than one third of the patients. In 2 patients hepatic cirrhosis became manifest after a completely asymptomatic period and in 2 others a lymphoproliferative disease appeared 2 and 8 years after the onset of purpura. In 51 / of patients the initial clinical pattern did not change In searching for a correlation between the development of nephropathy and cryoglobulin characteristics, none was demonstrated studying the cryoglobulin level, the presence of autoantibody and the complement components.

Introduction

The syndrome of essential cryoglobulinemia was described in full details by *Meltzer and Franklin* [15] in 1966. The peculiar symptom complex included dependent purpura, arthralgias, weakness. The same authors stressed the occurrence of rapidly progressive glomerulonephritis in 3 out of 10 patients. This finding was subsequently confirmed by several authors [1 3 4] More rarely were chronic active hepatitis or cirrhosis [4 6, 10, 13] and neurologic injuries [1 11] reported as complicating the course of the disease Despite the numerous reports on the incidence of renal lesions, the natural history of essential cryoglobulinemia is still uncertain and deserves further evaluation of large series. The present study of 35 (out of 56) patients with essential cryoglobulinemia followed for 4–13 years may provide some help in defining this important topic.

Patients

Significant cryoglobulinemia (cryoprotein values abov 80 mg l) was detected in 102 patients coming to our observation over period of 13 years. The cryoglobulinaemia was considered idiopathic in 56 patients. Among the patients with secondary cryoglobulinemia, 16 with Waldenström's disease were selected in order to compare their

8 Li C. Y Yam, L. T., and Lam, K. W.. Studies of acid phosphatase isoenzymes in human leukocytes. Demonstration of isoenzyme cell specificity J Histochem. Cytochem. *18* 901–910 (1970).
9 Lorbacher P.. Zur diagnostischen Anwendung des cytochemischen β-glucuronidase Nachweises in Gross and Van de Loo Leukämie (Springer Berlin 1972)
10 Meister H. und Fuchs, R.. Zytochemie der malignen Erythroblastose Folia haemat., Lz. *1* 71–80 (1972).
11 Mover S Li, C. Y and Yam L. T.. Semi-quantitative evaluation of tartrate-resistant acid phosphatase activity in human blood cells. J Lab clin. Med. *80* 711–717 (1972).
1 Shaw M T and Klemp V A. Cytochemical staining with oil red 0 and for β-glucuronidase in the diagnosis of acute leukaemia. Am. J clin. Path. *61* 169–172 (1974)
13 Woessner S. Lafuente Rodes, R and Sans-Sabrafen, J.. Valor del estudio combinado citoquímico e isoenzimático de la fosfatasa ácida en la identificación de la población blastica eritrémica. Sangre, Barcelona (in press).
14 Yam, L. T., Li, C. Y., and Lam, K. W.. Tartrate-resistant acid phosphatase isoenzyme in the reticulum cells of leukemic reticuloendotheliosis. New Engl. J Med. *284* 357–360 (1971)

Received December 28, 1977
Accepted April 22, 1978

S. Woessner MD
Department of Haematology
Hospital de la Cruz Roja,
Dos de Mayo 301 Barcelona (Spain)

Table I. Immunochemical classification of 44 essential mixed cryoglobulins

Immunochemical type	Cryoglobulin components	Number of cryoglobulins	Percentage
	IgG–IgM	24	54.5
Monoclonal mixed	IgG–IgA–IgM	7	15.9
(type II cryoglobulins)	IgG–IgA	1	2.3
Total		32	72.8
	IgG–IgM	7	15.9
Polyclonal mixed	IgG–IgA–IgM	3	6.8
(type III cryoglobulins)	IgG–IgG	2	4.5
Total		12	27.2

Monoclonal component

presence, in addition to IgG, of minute amounts of IgA.

Of the 16 patients with Waldenström's disease, 2 had type I, the others type II cryoglobulins. The IgM component was always of type k.

Clinical Studies

Age and sex (fig. 1): patients were 16–71 years old at the onset of symptoms, with a mean peak of incidence in the fourth decade. Female prevalence was observed. Only 2 persons, whose cryoglobulins were detected during a survey of cryoglobulinemic patients' relatives, were symptom free. The commonest initial symptom was purpura; arthralgias were present in about half of the patients. Leg ulcers were found in 5 patients, Raynaud's phenomenon in 8 and urticaria in –. Neurologic involvement presenting as paresthesia and numbness was only once observed.

The study population followed for a mean of 7 years (4–13 years) comprised 35 patients. The typical clinical pattern continued unchanged in a little more than 50% of these; also the 2 persons completely asymptomatic were symptomless after 5 and 8 years respectively (table II).

In 13 patients, proteinuria was detected 2–7 years after onset of symptoms. In 6 cases the nephropathy showed a progressive deteriorating course leading to renal insufficiency (creatinemia over 2 mg/dl) 3–8 years

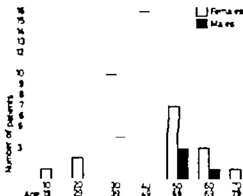


Fig. 1. Age at onset of initial symptoms in 36 patients with essential cryoglobulinemia.

clinical and serological data with those obtained in the group under study. The diagnosis of Waldenström's disease was based on the following criteria: the cytological feature of a pleomorphic infiltration of lymphocytes, plasma cells and lymphoplasmacytic cells together with the level of IgM (above 30 g/l).

Methods

Sera. Blood collected in a syringe warmed to 37 °C, was allowed to clot at 37 °C in a water bath. Serum was obtained after centrifugation at 800 *g* for 15 min at 37 °C.

Cryocrit. The amount of cryoglobulin in sera was evaluated by the cryocrit, a term indicating the volume percentage of whole serum occupied by the cryoprecipitate after centrifugation for 30 min at 800 *g* and at 4 °C of serum stored at 4 °C for 72 h.

Isolation of Cryoglobulins. The method described by *Meltzer and Franklin* [15] was followed.

Characterization of the Cryoglobulins. Washed cryoprecipitates were dissolved with PBS at 37 °C to the original volume of serum. The total protein was measured by the Lowry method [L]. Immunoelectrophoresis [17] was performed using commercially available antisera. The components of cryoglobulins were isolated by gel filtration on Sephadex G-200, using 0.1 *M* acetic acid-acetate buffer pH 3.

DNA Preparations. Calf thymus DNA (Sigma I highly polymerized) was used as source of DNA preparations. DNA concentrations were determined by measuring the optical density at 260 nm. ssDNA was prepared by heating solutions of 0.5 g/l for 12 min in a boiling water bath, followed by immediate cooling in an ice bath. dsDNA was obtained by chromatographic fractionation of a solution of 0.1 g/l in 0.001 *M* sodium monophosphate-sodium diphosphate buffer pH 6.8 on a column of hydroxyapatite; elution was accomplished stepwise by increasing gradients in molarity of the same buffer. The fraction eluted by 0.3 *M* buffer was recovered. ssDNA was iodinated as described by *Commerford* [2]. ^{14}C dsDNA from *Escherichia coli* internally labelled with thymine ^{14}C, was obtained from Amersham.

Serologic Studies. Antigammaglobulin activity was measured by the latex agglutination test (Latex Reagent RF, Behringwerke) and by the sensitized sheep cell agglutination test. Antibodies to ssDNA and dsDNA were determined by a modification of the Farr assay [21]. Specificity of the DNA binding activity was tested in inhibition studies with cold ssDNA and dsDNA by the method of *Picazo and Tan* [16].

Autoantibodies (antinuclear factor, smooth muscle, mitochondrial, gastric parietal cells autoantibodies) were investigated by the indirect immunofluorescent method using rat stomach, liver and kidney as antigen, and commercial fluoresceinated antiserums to human globulins (Hyland).

Complement Studies. C4 and Factor B were titrated by the lysis plate method according to *Lachmann et al.* [7]. C3 determination was performed by monodimensional simple immunoelectrophoresis according to *Laurell* [8]; the antisera were purchased from Behringwerke.

Results

Immunochemical characterization of the Cryoglobulins

In the group with the idiopathic form only 2 cryoglobulins with a single monoclonal immunoglobulin (IgG) were recognized, the other 54 being of the mixed type. In 44 of these we could classify immunochemically the purified immunoglobulins. 73% showed a monoclonal component (type II cryoglobulins), always an IgM type k with the only exception of 1 case with an IgA type k. 27% were polyclonal (type III cryoglobulins) (table I). Among the mixed ones the rheumatoid activity was bound to IgM in 41 cases (29 of type II and 10 of type III), IgA in 1 case (type II) and IgG in 2 (type III).

Cryoglobulins composed of more than 2 immunoglobulins were detected in 10 cases (7 of type II and 3 of type III) showing the

Table III. Cryoglobulin type, cryocrit and autoantibodies in essential cryoglobulinemia and in Waldenström disease

Diagnosis	Cryoglobulin type		Cryocrit % (mean values)	Autoantibodies			
	II	III		RF	ANA	dsDNA	ssDNA
Essential cryoglobulinemia	23/32	8/12	7.8	30/32 (245)[a]	2/25	0/20	2/20
Essential cryoglobulinemia with renal involvement	9/32	4/12	8.1	11/13 (240)	1/13	0/13	1/13
Waldenström's disease	14		48.3	13/14 (1,045)	0/10	0/10	0/10

RF = Rheumatoid factor ANA = antinuclear antibodies.
Number of patients with positive tests/total patients tested.
Mean titre highest dilution giving agglutination.

Table IV Levels (mean values) of complement components in essential cryoglobulinemia and in Waldenström's disease

Diagnosis	Number of patients tested	C3 mg/dl	C4	Factor B
Essential cryoglobulinemia	18	96	35%	115%
Essential cryoglobulinemia with renal involvement	10	74	7%	82%
Waldenström disease	6	76.5	29%	99%
Controls	20	110 ± 30	>65%	>65%

Functional titration percentage activity compared with that of pool of fresh normal human serum, considered as 100%.

and in 6 patients with Waldenström's disease. Depression of C4 level, though maximal in cryoglobulinemic patients with renal damage, was found in the three groups of patients. Factor B levels were in the normal range in all patients, so excluding a possible role of the alternate pathway in complement activation. C3 levels were found slightly depressed only in patients with renal insufficiency and in Waldenström's disease (table IV).

Discussion

In our series of essential cryoglobulinemias we have found, as others [3] that the most frequent type involved was the type II (mixed cryoglobulins with a monoclonal component). This is at variance with the finding of *Brouet et al* [1] who reported that of 29 essential mixed cryoglobulins only 2 were type II. The incidence of nephropathy in the present series followed up

Table II. Long-term follow-up (4–13 years mean 7 years) in 35 patients with essential cryoglobulinemia

Clinical course	Number of patients	Percentage
Asymptomatic	2	5 7
Uncomplicated Meltzer syndrome	16	45 7
Renal involvement (proteinuria ≥2 g/24 h)	7	20
Renal insufficiency (creatinemia ≥2 mg/dl)	6[1]	17
Hepatic cirrhosis	2	5 7
Chronic lymphocitic leukemia	1	2.7
Non-Hodgkin lymphoma	1	_7

- patients deceased

later 2 of these 6 patients received no therapy for their initial glomerular lesions the others were treated with steroids, in 2 in a combination with azathioprine In all patients with renal insufficiency a biopsy specimen showed diffuse glomerulonephritis with cell proliferation and endomenbranous deposits by immunofluorescence positive staining with anti IgG anti IgM and in four instances anti-beta 1C serums, along the basement membrane was shown

4 of these 6 patients are still living (1 is maintained on chronic hemodialysis) 4–12 years after the appearance of the purpuric syndrome while 2 have died 4 and 8 years after

In 2 patients whose liver was moderately enlarged at the first examination when the only complaints were those of the cryoglobulinemia and other signs of liver disease were inapparent splenomegaly appeared 4 and 8 years after the first purpuric rash of the legs. A liver biopsy was performed and the histologic study showed posthepatitic cirrhosis.

In 1 patient an 8-year period of purpura and arthralgia was followed by the onset of a chronic lymphocytic leukemia, and in another one a non-Hodgkin lymphoma was evident 2 years after purpuric lesions in the legs

Of the 16 patients of our series with Waldenström s disease only 1 presented signs of renal insufficiency 1 year after the initial diagnosis. The renal biopsy showed a membranous proliferative glomerulonephritis the immunofluorescence was negative

Serological studies

The high incidence of glomerular injuries in our patients prompted us to look for a possible correlation between renal involvement and some characteristics of the cryoglobulins. No difference was observed, as to the relative incidence of renal damage in the type – monoclonal or polyclonal – of the cryoglobulins. No correlation was found with the cryocrit, or with the titer of antigammaglobulin activity in the latex fixation test or with the presence of antinuclear factors There were no cases displaying antinative DNA antibodies while the finding of antibodies to denatured DNA was relatively frequent. Nevertheless, the reaction at least in most instances, was aspecific due to components other than immunoglobulins. It was specifically inhibited only in 3 patients with essential cryoglobulinemia 1 of these had renal insufficiency (table III) Other autoantibodies (smooth muscle, mitochondrial gastric parietal cell autoantibodies) were lacking in all the 3 groups of the patients examined

Measurement of serum complement components (C3 C4 and factor B) was performed in 28 patients with essential cryoglobulinemia (10 with renal involvement)

nological investigations of the factors operative in the bimodal pattern of evolution are needed.

References

1 Brouet, J C., Clauvel, J P Danon, F Klein, M and Seligman, M. Biologic and clinical significance of cryoglobulins. A report of 86 cases. Am. J Med 57 775–788 (1974).

2 Commerford, S. L. Iodination of nucleic acid in vitro. Biochemistry 10 1993–1999 (1971).

3 Cream, J J Clinical and immunological aspects of cutaneous vasculitis. Q Jl Med. 178. 255–276 (1976).

4 Feizi, T and Gitlin, N Immune complex disease of the kidney associated with chronic hepatitis and cryoglobulinemia Lancet ii. 873–876 (1969)

5 Gershwin, M. E. and Steinberg, A. D Qualitative characteristic of anti-DNA antibodies in lupus nephritis. Arthritis Rheum. 17 947–954 (1974)

6 Jori, G. P and Buonanno, G Chronic hepatitis and cirrhosis of the liver in cryoglobulinemia. Gut 13 610–613 (1972).

7 Lachmann, P J Hobart, M. J and Aston, W P Complement technology in Weir Handbook of experimental immunology (Blackwell, Oxford 1973).

8 Laurell, C B Quantitative estimation of protein by electrophoresis in agarose gel containing antibodies. Analyt Biochem 15 45–51 (1966).

9 Levinsky R. J Cameron, J S., and Soothill, J F Serum immune complexes and disease activity in lupus nephritis. Lancet i 564–567 (1977)

10 Levo, Y Gorevic, P D., Kassab, H J Zucker Franklin, D., and Franklin, E. C Association between hepatitis B virus and essential mixed cryoglobulinemia New Engl. J Med. 296 1501 1504 (1977).

11 Logothetis, J Kennedy W R., Ellington, A., and Williams, R. C. Cryoglobulinemia neuropathy incidence and clinical characteristics. Archs Neurol 19 389–397 (1968)

12 Lowry O H Rosebrough, N J Farr A. L., and Randall, R. J Protein measurement with the Folin phenol reagent. J biol. Chem. 193 265–275 (1951).

13 Mathison, D. A. Condemi, J J Leddy J P Callerame, M. L., Panner B. J and Vaughan, J H. Purpura arthralgia and IgM IgG cryoglobulinemia with rheumatoid factor activity Response to cyclophosphamide and splenectomy Ann. intern. Med. 74 383–390 (1971)

14 McIntosh, R. M., Koss, M. N., and Gocke, D J The nature and incidence of cryoproteins in hepatitis B antigen (HB Ag) positive patients Q. Jl Med. 40 23–38 (1976)

15 Meltzer M. and Franklin, E. C. Cryoglobulinemia. A clinical and laboratory study II. Cryoglobulins with rheumatoid factor activity Am. J Med. 40· 837–856 (1966).

16 Picazo, J J and Tan, E. M.; Specificities of antibodies to native DNA. Scand. J Rheumatol. 11 35–41 (1975).

17 Scheidegger J J Une micro-méthode de l'immuno-électrophorèse. Int. Archs Allergy appl. Immun. 7 103–110 (1955).

18 Soothill, J F and Steward, M. W The immunopathological significance of the heterogeneity of antibody affinity Clin exp Immunol. 9 193–200 (1971).

19 Stone, M J and Fedak, J E.. Studies on monoclonal antibodies. II Immune complex (IgM IgG) cryoglobulinemia. the mechanism f cryoprecipitation. J Immun. 113 1377 1385 (1974)

20 Takahashi, M Czop, J Ferreira, A and Nussenzweig, V Mechanism of solubilisation of immune aggregates by complement. Implications for immunopathology Transplants Rev 32 121 139 (1976).

21 Wold, R. T Young, F E Tan, E. M and Farr R. S Deoxyribonucleic acid antibody· method to detect its primary interaction with deoxyribonucleic acid Science 161 806–807 (1968).

Received, February 23, 1978
Accepted, July 1, 1978

Dr F Invernizzi, Via San Vincenzo 4, I-20123 Milano (Italy)

for a long period (mean 7 years) is a little higher than that of other large series [1 3 15] Our cases were equally distributed among type II and III as found by *Cream* [3]

We have found no correlation between the development of renal damage and any of the cryoglobulin characteristics investigated In effect no significant difference was found in autoantibody titer or in complement levels between the patients with or without renal lesions The same negative conclusions may be drawn from studies on the presence of anti DNA antibodies. Particularly striking is the absence of correlations between the cryocrit levels and the presence of symptoms related to cryoglobulinemia During the observation period the cryocrit did not change beyond limited fluctuations not statistically significant, as though every patient appeared to present a peculiar cryoprecipitate level In our study the presence of a cryoglobulin appeared to have little modified the usual course of Waldenström's disease actually only 1 of the 16 patients presented a glomerular lesion in this context *Stone and Fedak* [19] concluded that, except for its effect on serum viscosity the prolonged cryoglobulinemia of Waldenström's disease did not result in any detectable adverse consequences. On the other hand, others [1] have found a worsening in the basic disease

In searching for a correlation with the appearance of renal complications it is possible that the study of mixed cryoglobulins as antigen-antibody complexes may give useful information, though there is no agreement on the characteristics of the immune complexes determining renal lesions either the size of the complexes [9] or the antibody affinity [5 18] or the availability of complement components having a solubilization activity on the complexes [20] Our finding of a progression toward hepatic cirrhosis is of interest because such a complication appeared without signs of hepatic involvement until the development of a splenomegaly Such cases, similar to those described [6 13] together with the presence of HBsAg in some cryoprecipitates [14] raise the important question of what comes first: the hepatic damage or the cryoglobulin

In this context we can include the 2 patients in which a lymphoproliferative disease developed 2 and 8 years after the onset of symptoms related to cryoglobulins. The interpretation of such cases may moreover be controversial because we cannot exclude the possibility that the lymphoma, though not detectable, preceded the onset of cryoglobulinemia others [1] have indeed considered the cryoglobulinemia as secondary to lymphoma. The explanation we favor is that both cryoglobulins and lymphoproliferation are secondary phenomena to the same basic immunoproliferative disorder

In spite of this high frequency of complications, in a greater proportion of patients (51%) the disease did not show such a *downhill course and 18 persons* after a follow up of more than 4 years exhibit the same initial symptom complex 2 of these remain completely symptom free

It is apparent from our data that the clinical course of essential cryoglobulinemia falls into two groups characterized by the progression or not of the vasculitic lesion to organs other than the skin If one considers that, in addition to such complications, the *effectiveness of the various agents proposed* in the treatment of this condition – steroids and cytotoxic drugs – is far from established. the conclusion is that further immu

port another case of primary plasmacytoma f lymph nodes. Cervical, mediastinal and abdominal lymph nodes were involved, but repeated examinations did not show bone marrow involvement. When the patient died, 17 months after the first symptoms and 9 months after diagnosis, the autopsy disclosed huge lymph node tumours, but the bone marrow was still not involved.

Case Report

M. H white male born 1919 had routine X-ray f the chest in August 1973 the mediastinum ppeared enlarged. Physical examination revealed no splenomegaly or lymphadenopathy. Laboratory studies were normal, except for impaired renal function (creatinin 255 μmol/l) and proteinuria (3.1 g/l). Intravenous pyelography disclosed small kidneys with lmost normal function.

I January 1974 the mass in the mediastinum had increased. A small lymph node in the left axilla was biopsied, revealing diagnosis of sinus histiocytosis. The patient was referred to our hospital in April 1974. Small axillary and inguinal lymph nodes were then palpable and in the left supraclavicular fossa mobile non-tender firm node of 2 cm was found. The liver was felt 3 cm under the right costal margin, but the spleen was not palpable. Thorough examination of the upper air passages was unremarkable. Laboratory studies showed ESR 17 mm/h, Hb 12.5 g/dl, WBC 9.2 × 10⁹/l with 65% neutrophils, and platelets 224 10⁹/l. Liver functions and serum proteins were normal (*total protein 79 g/l, γ-globulin* 16.5 g/l). The renal function was impaired (creatinine 260 μmol/l with clearance of 26 ml/min). Serum immunoglobulins (Mancini technique) were IgG 17.1 g/l, IgA 1.9 g/l, IgM 1.6 g/l. Planimetry of the electrophoresis pattern (after identification of the peak by specific antisera) showed the presence of 1.5 g/l kappa light chains in the serum. A proteinuria of 8–13 g/24 h was found, 85% of which consisted of kappa light chains (demonstrated gain by planimetry of the electrophoresis pattern after identification of the specific peak with anti-kappa antiserum). Biopsy of the lymph node in the left supraclavicular space gave diagnosis of plasmacytoma. Bone marrow aspiration (sternum) showed 2.6% plasma cells, not grouped in nests; bone marrow biopsy (iliac crest) gave similar picture, which was considered normal. A liver biopsy specimen (Menghini technique) showed no infiltration with plasma cells. A skeletal survey was normal, but lymphographic studies showed left para-aortic mass of lymph nodes, lmost 10 cm in diameter. Technetium scanning revealed hepatomegaly but no splenomegaly.

Radiotherapy to the mediastinal mass (4,000 rad in 20 days) had no beneficial effect. Chemotherapy with low dose alkylating agents also failed to reduce the tumour masses or the proteinuria. I October 1974 pancytopenia occurred (Hb 5.6 g/dl, WBC 0.9 × 10⁹/l and platelets 14 × 10⁹/l). A bone marrow aspirate (iliac crest) showed megaloblastic erythropoiesis, but normal myeloid series. Plasma cells were 4%, but no immature plasma cells or grouping of plasma cells was observed. Cytostatic therapy was replaced by corticosteroids and the patient made quick recovery and resumed work. However in January 1975 he w *readmitted with signs of severe bronchopneumonia.* Liver and lymph nodes were unchanged. Laboratory studies showed ESR 140 mm/h. Hb 10.0 g/dl, WBC 9.5 × 10⁹/l and platelets 120 × 10⁹/l. Renal function, serum immunoglobulins and proteinuria were unchanged. The patient died from respiratory failure the day after admission. Bone marrow aspirated immediately post-mortem (iliac crest), revealed 3% plasma cells. An autopsy was performed.

Pathology

Lymph Nod The normal architecture of the lymph node was completely lost (fig 1) and replaced by diffuse proliferation of typical plasma cells (fig. 1 inset). A few Russell bodies were seen and there was capsular infiltration. The plasma cells were negative in the periodic-acid Schiff (PAS) stain, and orange-red in the trichromic stain of Ladewig, suggesti of only light chain production [*K. Lennert* pers. commun.].

Autopsy All lymph nodes examined from various sites (inguinal, iliac, para-aortic, mediastinal, supraclavicular and in the porta hepatis, fig 2) showed the histological picture described for plasmacytomas. The kidneys had the typical structure of myeloma kidney nd they showed infiltration

Acta haemat 61 100–105 (1979)

Primary Plasmacytoma of Lymph Nodes

A Case Report

J Jansen and P Blok

Departments of Medicine and Pathology Free University Hospital, Amsterdam

Key Words. Plasmacytoma Lymph node Myeloma Mediastinal mass

Abstract. Most plasmacytomas of lymph nodes are secondary metastases from myeloma or from primary plasmacytoma of the upper air passages. Primary plasmacytomas of lymph nodes are very rare

A case of primary plasmacytoma of mediastinal cervical and para aortic lymph nodes is reported. Bence Jones proteinuria of 8 g/24 h was present. Repeated bone marrow examinations were normal When the patient died 17 months after the first symptoms, the bone marrow was still not involved.

Introduction

Plasma cells are derived from lymphocytes, which are thought to be derived from the human equivalent of the bursa of Fabricius in chickens [24] Under normal conditions plasma cells are mostly located in lymph nodes, respiratory and digestive tracts and bone marrow [24 26] Plasmacytomas may therefore be expected at all these locations, but – for unknown reasons – they rarely occur outside the bone marrow [2, 6 26]

The most typical plasmacytomas are myelomas, involving the bone marrow and presenting either as tumour nodules (multiple myeloma) or as diffuse myelomatosis [2, 26] Extramedullary plasmacytomas are most frequently secondary metastases from myelomas [21] but primary extramedullary plasmacytomas do occur [6 26] These tumours are mostly located in the upper air passages, but various locations have been reported [6 26] Like other malignancies, these tumours can metastasize to drainage lymph nodes to bone and to bone marrow [6 26] The enlargement of cervical lymph nodes has been the first symptom of a plasmacytoma of the upper air passages [8] The bone lesions are sometimes indistinguishable from myeloma but are more often typified by only a few large osteolytic deposits [26]

Primary plasmacytomas of lymph nodes are extremely rare Various reviews [9 20 26] did collect no more than 12 cases, many of which were not fully documented We re

Table I. Cases of primary plasmacytoma of lymph nodes

Author	Sex/age	Presenting site	Bone marrow (% plasma cells)	X-skeleton	M-protein in serum	Bence Jones proteinuria	Therapy	Survival from start of symptoms	Survival from diagnosis	Remarks
Kleinmann [14]	M/56	neck	neg. (n.s.)	neg.	neg.	neg.	ACTH	31+m	12+m	well and alive with persistent disease
Sutton et al. [25]	M/66	neck	neg. ( )	neg.	neg.	neg.	surgery	24+m	22 m	well and alive after resection of recurrence
Morgenfeld et al. [18]	F/64	neck	neg. (3%)	neg.	pos.	neg.	surgery radiother chemother	14+m	10+m	well and alive [illegible]th [illegible]illary recurrence
Nelson and Lyons [20]	M/32	neck	neg. (1.5%)	neg.	neg.	neg.	surgery radiother	26+y	17+y	well and alive after local recurrences
Gaston et al [9] (case 2)	M/67	neck	neg. (2.5%)	neg.	neg.	neg.	surgery	19+m	1[illegible]+m	[illegible]ell and alive
Nakakawa et al [19]	M/55	hilar	neg. (2.5%)	neg.	neg.	neg.	surgery	4+m	4+m	[illegible]ell and alive
Jensen and Blok	M/55	mediastinal	neg. (2.6%)	neg.	kappa light chain	8 g/24 h	radiother chemother	17 m	9 m	died from broncho-pneumonia metastasis to kidneys

neg. = Negative pos. = positive = still alive when reported = examined after removal of the tumour
m = months y = years, n.s. = not stated.

with some scattered groups of plasma cells. The bone marrow (vertebral column, superior anterior spines of the iliac crest, and ribs) showed no infiltration with plasma cells. The lungs had bilateral bronchopneumonia with abscesses. Larynx, pharynx, tongue and digestive tract were unremarkable. Stains for amyloid were negative.

Discussion

It is not well known why plasmacytomas almost exclusively start inside the bone marrow, whereas other tumours of B-lymphocytes most often start inside lymph



Fig. 1 The normal architecture of the lymph node is completely replaced by proliferations of plasma cells (inset). HE. × 50 (inset × 1,300)

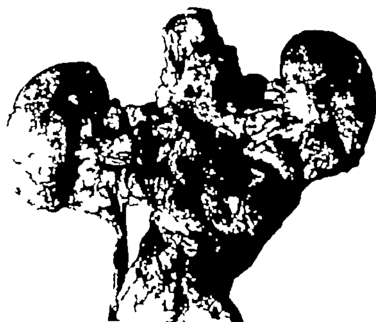


Fig. 2. Huge para-aortic tumour masses at autopsy

Table I. Cases of primary plasmacytoma of lymph nodes

Author	Sex/age	Presenting site	Bone marrow (% plasma cells)	X-skeleton	M-protein in serum	Bence Jones proteinuria	Therapy	Survival from start of symptoms	Survival from diagnosis	Remarks
Kleemann [14]	M/56	neck	neg. ( )	neg.	neg	neg	ACTH	31 m	12+m	well and alive with persistent disease
Saxon et al [25]	M/66	neck	neg. ( )	neg.	neg.	neg	surgery	24 m	22+m	well and alive after resection of recurrence
Morgenfeld et al [18]	F/64	neck	neg. (3 %)	neg	pos.	neg	surgery radiother chemother	14 m	10+m	well and alive with axillary recurrence
Nelson and Lyons [20]	M/32	neck	neg. (1.5 %)	neg	neg	neg	surgery radiother	26+y	17+y	well and alive after local recurrences
Gaston et al [9] (case 2)	M/67	neck	neg. (2.5 %)	neg	neg.	neg	surgery	19+m	12+m	well and alive
Namikawa et al [19]	M/55	hilar	neg (2.5 %)	neg.	neg	neg	surgery	4 m	4+m	well and alive
Jensen and Blok	M/55	media-stinal	neg (2.6 %)	neg.	kappa light chain	8 g/24 h	radiother chemother	17 m	9 m	died from broncho-pneu-monia metastasis to kidneys

neg = Negative pos. = positive = still alive when reported = examined after removal of the tumour m = months y = years s. = not stated.

with some scattered groups of plasma cells. The bone marrow (vertebral column, superior anterior spines of the iliac crest, and ribs) showed no infiltration with plasma cells. The lungs had bilateral bronchopneumonia with abscesses. Larynx, pharynx, tongue and digestive tract were unremarkable. Stains for amyloid were negative.

Discussion

It is not well known why plasmacytomas almost exclusively start inside the bone marrow whereas other tumours of B-lymphocytes most often start inside lymph



Fig. 1 The normal architecture of the lymph node is completely replaced by proliferations of plasma cells (inset) HE. × 50 (inset × 1,300)

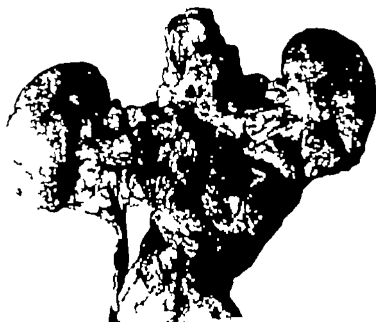


Fig. 2. Huge para-aortic tumour masses at autopsy

poietic system. Am J clin. Path. 20 934–945 (1950)
5 Conklin, R. and Alexanian, R. Clinical classification of plasma cell myeloma. Archs intern. Med. 135 139–143 (1975).
6 Dolin, S. and Dewar J P Extramedullary plasmacytoma. Am J Path. 32. 83–103 (1956).
7 Durie, B. G M. and Salmon, S. E. A clinical staging system for multiple myeloma. Cancer 36 842–854 (1975).
8 Fishkin, B G and Spiegelberg, H. L. Cervical lymph node metastasis as the first manifestation of localized extramedullary plasmacytoma. Cancer 38 1641 1644 (1976).
9 Gaston, E. A Dollinger M. R. Strong, E. W and Hajdu, S. I. Primary plasmacytoma of lymph nodes. Lymphology 1 7–15 (1969).
10 Guesch, J La forma ganglionar forma rara del plasmocitoma extramedular Sangre, Barcelona 11 249–251 (1966)
11 Gupta, R. M., Roy D C., Gupta, L M., and Khanna, S. Extramedullary plasmacytoma IgG type I presenting as mediastinal syndrome Br J Dis. Chest 68 65–70 (1974).
12 Jackson, H Parker F and Bethea, J M. Studies of diseases of the lymphoid and myeloid tissues. II. Plasmatocytomata and their relation to multiple myelomata. Am. J med Sci 181 169–180 (1931)
13 Jáquier-Doge, E., Nicod, J L. et Chapuis, J P Un cas de plasmocytome à localisation ganglionnaire observé cliniquement pendant plus de 6 ans et contrôlé anatomiquement Schweiz. med. Wschr 77 186–190 (1947).
14 Kleimann, M. Plasmocitoma ganglionar Prensa méd argent. 43 1702–1705 (1956).
15 Kyle, R A. Multiple myeloma, review of 869 cases. Mayo Clin. Proc 50 29–40 (1975).
16 Lejeune, F Turpin, F Lecouturier J P Bouvier J B. et Lortholary P Plasmocytome ganglionnaire. Nouv Presse méd. 1 1065–1068 (1972).
17 Maresch, R. Über ein plasmazelluläres Lymphogranulom. Verh. dt. path. Ges. 13 257–263 (1909).
18 Morgenfeld, M. C., Ferral, N L., and Garnek, L. Plasmocitoma de inciciacion ganglionar Sangre, Barcelona 12. 45–52 (1967).
19 Namikawa, S., Hata, H., Nunomi, F Yusa, H., and Kubo, K. A case of intrathoracic plasmacytoma. Jap. J thorac. Surg. 25 357–362 (1972).
20 Nelson, M. G and Lyons, A. R. Plasmacytoma of lymph glands Cancer 10 1275–1280 (1957).
21 Pasmantier M. W and Azar H. A. Extraskeletal spread in multiple plasma cell myeloma Cancer 23 167–174 (1969).
22 Rowlands, B. and Shaw N. Extramedullary plasmacytoma. Br med. J ii. 1302 1303 (1954).
23 Simon, M. A. and Eldlow E. Plasmacytoma of lymph node. New Engl J Med 243 335–338 (1950).
24 Stites, D. P and Caldwell, J L. Phylogeny and ontogeny of the immune response; in Fudenberg, Stites, Caldwell and Wells, Basic and clinical immunology pp. 117–129 (Lange Medical Publications, Los Altos 1976).
25 Sutton, L., La Rosa, J and Linn, B. Plasmacytoma of lymph nodes. J Am. med. Ass. 197 294–296 (1966).
26 Wiltshaw E. The natural history of extramedullary plasmacytoma and its relation to solitary myeloma of bone and myelomatosis. Medicine, Balt 55 217–238 (1976).

Received: February 15, 1978
Accepted: May 25, 1978

J Jansen, MD, Department of Haematology
(Isolation Pavilion, Bldg. 16),
Leiden University Hospital, Leiden
(The Netherlands)

nodes Perhaps different types of B lymphocytes are involved as has been suggested for medullary and extramedullary plasmacytomas [26]

Peripheral lymphadenopathy is a rare finding in myeloma at diagnosis [15 25] In advanced cases infiltration of lymph nodes can be found in up to 40% of cases at autopsy [4 21] but this involvement is mostly only microscopical [21] It is a metastatic, near terminal event [21] and seldom gives rise to gross enlargement of peripheral lymph nodes [4]

To come to a diagnosis of primary plasmacytoma of lymph nodes, first the malignant nature of the tumour had to be established, because non-malignant plasma cell granulomas can be confused with malignant plasmacytomas [2 26] The completely lost architecture of the lymph nodes the capsular infiltration and the metastasis to the kidneys established the malignant nature of our case Secondly it had to be proven that the plasmacytomas of the lymph nodes were not secondary to myeloma or to a plasmacytoma of the upper air passages. Repeated investigations during life and finally the autopsy did not demonstrate a primary site but the lymph nodes

It is noteworthy that in this case a large amount of Bence Jones protein, reported to be diagnostic of myeloma [5] was found A gross calculation of the number of tumour cells from the data of *Durie and Salmon* [7] demonstrated that this large amount of Bence Jones protein can be accounted for completely by the huge tumour masses found at autopsy Furthermore, it is noteworthy that the normal serum immunoglobulins were not depressed in this patient with such a big load of malignant plasma cells.

At least 6 well-documented cases of primary plasmacytoma of lymph nodes have been published up to now (table I) and probably other cases [1 3 12, 13 16 23] (not enclosed in table I because they were not fully documented) also belong to this group of tumours. Furthermore, some cases with discrete bone or bone marrow involvement [11 12 22] perhaps also represent cases of primary plasmacytoma of lymph nodes.

Thus there exists a small group of tumours, showing histologically and cytologically the picture of neoplasms of plasma cells, but behaving clinically different from myeloma. In fact it could be argued that this group of plasma cell tumours should be included in the classifications of malignant lymphomas Especially the cases of *Maresch* [17] *Morgenfeld et al* [18] and our case behaved rather like malignant lymphoma than like myeloma

Acknowledgements

We are grateful to Dr *E. Willshaw* (London) for her critical advice, to *Y. Isozaki* for the translation of reference 19 to *J Mulder* for immunochemical help and to Ms. *A H Jansen* for secretarial assistance

References

1 Andersen, P E. Extramedullary plasmacytomas. Acta radiol scand. *32* 365–374 (1949)
2 Azar H A.. Pathology of multiple myeloma and related growths, in Azar and Potter Multiple myeloma and related disorders, vol. I pp. 1–85 (Harper & Row Hagerstown 1973).
3 Basset, A et Scapler J Sur un cas d'un plasmocytome cervical. Monde méd. *47* 7–8 (1937).
4 Churg, J and Gordon, A. J Multiple myeloma. Lesions of the extraosseous hemato-

screening in investigations dealing with creening unselected populations has seldom been based on the degree of severity or the aetiology of anaemia, these aspects are treated in the present study It presents (1) the haematocrit values, (2) the prevalence of anaemia, and (3) the aetiology of anaemia in a middle-aged population according to age and sex.

Material and Methods

The subjects consisted of the middle-aged (40–64 years) population of the Säkylä and Koylio municipalities (2,431 people) in Southwest Finland. Although total of 1,223 (93.2%) of the women and 1,045 (93.4%) of the men participated in the multiphasic screening programme, carried out between 9 September 1973 and 11 January 1974 [24], the participation percentage in the anaemia screening was 93.1 for women and 93.3 for men because 2 of the women refused to give blood sample and 1 man dropped out

Determination of Haematocrit Value

In the determination of the haematocrit value, 2 ml of blood were collected from the ulnar vein into an EDTA tube, the compression applied t the sitting subject being as light as possible. After the sampling, the tube was placed in mixer and rotated for 10 min. Then samples were drawn into two heparinized capillary tubes which were sealed with plastic and centrifuged in Clay Adams Autocrit CT 2905 for 5 min. The erythrocyte count was made under the magnifying glass of the Autocrit, and the mean value of the two samples was recorded as the result in whole percentage units.

Aetiological Clarification of the Anaemias

When the haematocrit value was $< 35\%$ in women and $< 38\%$ in men, the sedimentation rate of the blood sample was determined and blood film was made, which was examined microscopically by haematologist The physician (U T.) in charge then carried out general examination of the subjects As the subject' history was recorded, special attention was paid to factors significant to the aetiology of anaemia [8], factors such as various alimentary tract symptoms, the absence of main sources of iron in food, the use of analgesics, previously verified anaemia, diseases causing anaemia, previous operations, and, for women, menstruation, menorrhagia, child births and abortions These data were obtained with the aid f questionnaire prepared for this purpose.

When the cause of the anaemia could not be determined by general examination (the aetiology being other than menorrhagia, bleeding haemorrhoids or chronic rheumatoid arthritis), the patient was referred to the hospital for further investigation Iron deficiency as the cause of hypochromic anaemia (microscopy of a stained peripheral blood smear or calculation of the red cell indices) was determined by demonstration of reduced iron content of the marrow or hypoferraemia with an elevated serum binding capacity and by positive response to orally given iron [20] (the rise in haematocrit fter 3 weeks therapy at least 8% in those cases where it had been $<30\%$ initially and at least 5% in those where it had been 33–34%). The criteria applied for menorrhagia were those of *Beveridge et al.* [3].

Results

Table I demonstrates that there was a clear increase with age in the mean haematocrit values of the women, whereas men showed a slight decrease with age In all age groups the mean values for men were higher than the corresponding values for women. Table II presents the prevalence of anaemia according to age and sex, the criteria being $HCT < 35\%$ for women and $HCT < 40\%$ for men [8] The prevalence decreased with age among the women and increased with age among the men.

Table III shows the anaemias found in the women ($HCT < 35\%$) and men ($HCT < 38\%$) according to the aetiological distribution of *De Gruchy* [8] (The criteria applied in the table for men deviates from that in table II because the slightly anaemic

Acta haemat. 61 106–113 (1976)

Screening for Anaemia in a Middle-Aged Population in Southwest Finland

Jorma Takala

Department of Public Health, University of Turku, Turku

Key Words. *Anaemia Middle-aged population Screening*

Abstract. The occurrence and aetiology of anaemia were investigated and the benefit of screening for anaemia was evaluated in a middle aged population (2,431 persons aged 40–64 years) in Southwest Finland A total of 93% of the persons participated in the anaemia screening

The overall prevalence of anaemia was 2.1 and 2.8% for the women and men respectively when the haematocrit values of <35% for women and <40% for men were used as the criteria for anaemia The majority (85%) of the anaemic women (HCT <35%) were hypoferric, chronic blood loss being the main aetiological factor in all cases. So-called secondary anaemia was confirmed in 15% of the cases Half of the anaemias (HCT < 38%) in men were due to iron deficiency caused by chronic blood loss, and one third were secondary anaemias. Half of the anaemic men over 50 years of age were being treated by a doctor for the causative disease or for some other chronic disease Moreover since untreated anaemia in men under 50 years of age was relatively rare (1 1%) a systematic screening of middle-aged men for anaemia is not recommended. The majority (74%) of the anaemias caused by menorrhagia in women under 50 years of age were mild (HCT 30–34%) and mild anaemia has not been shown to constitute any substantial health hazard Since on the other hand, untreated anaemia in the women over 50 was relatively rare (0 6%) a systematic screening of middle aged women for anaemia is also considered inappropriate.

Introduction

Anaemia is one of the most common of physician's findings [4 14] Its importance from the general practitioner's point of view has been stressed because, although easy to treat, it may cause a substantial impairment of general health if left untreated [15] It has been assumed that even mild anaemia weakens a person's general health status [33] But this hypothesis has not been demonstrated with certainty [11] and both the significance of anaemia to public health and the benefit of screening for it are still disputable.

Since the evaluation of the benefit of

Table III. Aetiology of the anaemia found i the examined men and women aged 40–64 in two rural districts Southwest Finland (anaemia HCT < 35 % women, HCT < 38 % men)

Aetiology of anaemia	Women				Men			
	40–49	50–59	60–64	total	40–49	50–59	60–64	total
Post-haemorrhagic anaemia								
Acute								
Chronic (iron deficiency anaemia caused by chronic blood loss)	19	3		22	1	3	1	5
Impaired red-cell production								
Due to deficiency of substances essential for erythropoiesis								
Alimentary iron deficiency anaemia								
Megaloblastic anaemia due to deficiency of vitamin B_{12} or folic acid						1		1
Disturbance of bone-marrow function due t various causes								
Infection								
Renal failure						1		1
Chronic liver disease								
Malignant tumour								
Aplastic anaemia								
Collagen disease		2	1	3		1		1
Bone-marrow infiltration			1	1		1		1
Hypothyreosis				–				
Increased red-cell destruction								
Haemolytic anaemia due to corpuscular defects							1	1
Haemolytic anaemia due to extracorpuscular causes								
Total	19	5	2	26	1	7	2	10

the same manner as in some other studies [7 13 21 23] The men showed a slight decrease in the mean values from the youngest to the oldest age groups. Generally speak ing, either a slow decrease has been found in the mean values of men starting from the age of 20–34 [21 23 27] or no consistent decrease has been demonstrated [7 17 18, 22] Notwithstanding the fact that the investigations concerned differed in such aspects as selection of material dropouts, and, in part also, in methods applied, the differ ences between the mean values of haematocrit, as well as the values for the prevalence of anaemia, are surprisingly slight. Howev er in the present study anaemia was twice as common in men under 60 as in *Takkunen* s study [27] of a Finnish adult population. In contrast the differences between the women in these two studies were few As in some other investigations the prevalence of anaemia decreased in the present study

Table I. Haematocrit values of the examined men and women aged 40–64 in two rural districts in Southwest Finland

	Women						Men					
	40–44 (n = 261)	45–49 (n = 300)	50–54 (n = 235)	55–59 (n = 213)	60–64 (n = 212)	total (n = 1221)	40–44 (n = 277)	45–49 (n = 251)	50–54 (n = 221)	55–59 (n = 141)	60–64 (n = 154)	total (n = 1044)
Mean value of haematocrit	40.[illegible]	41.1	41.4	42.[illegible]	42.4	41.5	45.7	45.6	45.3	45.3	45.1	45.4
Standard deviation	3.23	3.03	3.03	3.15	[illegible].94	3.15	2.63	2.89	4.60	3.42	3.59	3.44

Table II. *Prevalence of anaemia in the examined* men and women aged 40–64 in two rural districts in Southwest Finland (anaemia HCT < 35 % women, HCT < 40 % men)

Age	Women			Men		
	number examined	HCT < 35 %		number examined	HCT < 40 %	
		n	%		n	%
40–49	561	19	3.4	528	8	1.5
50–59	448	5	1.1	362	15	4.1
60–64	212	2	0.9	154	6	3.9
Total	1,221	26	2.1	1,044	29	2.8

men (HCT 38–39%) were not examined by a physician to clarify the aetiology of their anaemia.) 22 women (85%) had iron deficiency anaemia and 4 (15%) suffered from so-called secondary anaemia. In all cases, the main cause of iron deficiency anaemia in women was chronic blood loss, menorrhagia being involved in 20 cases and other uterine bleeding in 2. The iron deficiency anaemia of 1 woman was attributable to both menorrhagia and clear scarcity of iron *in her daily food*. The secondary anaemia was due to rheumatoid arthritis in 3 women *and to myeloma in 1. Half of the anaemias* (HCT < 38%) found in men were due to iron deficiency while secondary megaloblastic, and haemolytic anaemia were present in 3, 1 and 1 men respectively. *In all cases* the cause of iron deficiency anaemia in men was chronic blood loss, caused by haemorrhoids in 3 cases and by gastric ulcer in 1. In a man with osteoarthritis a probable alimentary bleeding due to indomethacin was involved. The 3 cases of secondary anaemia in men were due to chronic rheumatoid arthritis, diabetic glomerulosclerosis associated with substantial renal failure and chronic lymphatic leukaemia, respectively. In the case of haemolytic anaemia, paroxysmal nocturnal haemoglobinuria was involved. The aetiology of the megaloblastic anaemia remained unclear because the patient died within 24 h after being admitted to hospital. On the basis of necropsy chronic pyelonephritis with related papillnecrosis was considered the primary cause of death.

Discussion

In the present study the mean haematocrit values increased with age in women in

mia [8] a systematic screening, according to the present study may not be appropriate for women over 50 years of age (the prevalence of untreated anaemias being 0.6%), nor for men under 50 (the prevalence of untreated anaemias being 1.1%), because the determined prevalences were low for the screening of a condition whose significance to public health is unclear. Irrespective of a low prevalence, screening for anaemia would be rewarding if it enabled severe but still curable diseases such as cancer to be verified early; however, it does not [9].

The benefit of screening men over 50 years of age for anaemia is disputable, even though the prevalence is relatively high. About half (11/21) of the anaemic men in this age group of the present study were already being treated by a doctor for the disease which had caused the anaemia or for some other chronic disease.

With regard to men, the benefit of screening for anaemia may be studied from another point of view also. Since the majority of anemias in men are caused by iron deficiency due to haemorrhage from the alimentary tract, a gastrointestinal examination is generally essential [6]. Because the majority of the present anaemias were mild with slight symptoms, at least 2% of the male subjects would have required further hospital examination to clarify the aetiology of the anaemia. It should be clear that currently no possibilities exist for making the great number of diagnostic investigations that would be needed if the screening for anaemia were to be extended to cover all middle-aged men. If, again, the criterion for anaemia were to be made more stringent, the prevalence of anaemia in regard to benefit would become low: for instance in the present study the criterion HCT < 38% showed only 0.8% of the men as having untreated anaemia.

In addition, the benefit of a systematic screening for anaemia in women under 50 years of age proved doubtful because the majority (14/19) of the anaemias due to menorrhagia in these women were mild (HCT 30–34%) and, as such, do not constitute a significant health hazard.

Instead of employing a systematic screening, there would be a possibility to conduct screening for anaemia in connection with health centre visits and screening for cervical cancer and to direct such screening toward the iron deficiency risk groups, i.e. towards women suffering from abundant menstruation, men suffering from haemorrhoids or from diverse symptoms referable to the alimentary tract and men using abundant analgesics. The cheapest and probably also the most effective means of reducing the number of women suffering from iron deficiency anaemia would be to organize a special follow-up programme in health centres for women who have previously been treated for anaemia, for according to some investigations [3, 15] iron deficiency anaemia has been found to relapse in women in the majority of cases. Even in the present study, with the exception of 1 case, all women suffering from moderately severe anaemia (HCT < 30%) had already been treated for anaemia earlier.

References

1 Acheson, R. M.: Thoughts on service for the pre-symptomatic diagnosis of disease. Publ. Hlth 77: 261–273 (1963).

2 Aromaa, A.: Terveystarkastukset ja seulontamenetelmät. In J. Aer: Kansanterveystyön käsikirja (Tammi, Helsinki 1975).

among middle aged women and increased among middle aged men [18 21 23 27]

The commonest form of anaemia is that caused by iron deficiency [14 15 17] due to chronic blood loss [4 20] In women such a blood loss usually derives from menorrhagia [3 15] while men primarily suffer from haemorrhage from the alimentary tract [3 15 17] which according to an extensive study on a hospital population [3] is due to haemorrhoids, gastric or duodenal ulcer the use of salicylates or diverticulosis. In the present study too iron deficiency anaemia was the commonest form of anaemia in both women and in those men whose haematocrit value was less than 38% The most significant cause of iron deficiency anaemia was menorrhagia in women and haemorrhoids in men

Efforts were made to determine, on the basis of the questionnaire used in the screening the aetiology of anaemia in slightly anaemic men (HCT 38–39%) who had not previously been examined. In about half of the cases (10/19) the data indicated that the aetiology of the anaemia was caused by iron deficiency due to chronic blood loss 6 men had reported that they used analgesics and consequently there existed a possibility of latent haemorrhage from the alimentary tract. 3 men had haemorrhoids, and 1 had a duodenal ulcer The causes of the rest of the slight anaemias (9/19) remained unknown

A contributory factor to a negative iron balance especially in women is insufficient iron in food. It has been shown in haematological investigations and nutritional interviews of various groups that women and men with iron deficiency eat less meat or prepared meat foods than do those without iron deficiency [26] In the present study with the exception of 1 woman no clear shortages of the main sources of iron in food were found for either sex

Even though anaemia has been shown to meet most of the requirements set for a disease to be screened [1 13] the benefit of screening for this disorder has been doubted [13 19] In fact, anaemia does not indisputably meet the requirement presented by *Wilson and Jungner* [30] according to which a disease to be screened should be a significant public health problem [11 28]. When the significance of this kind of a problem is evaluated suitable criteria are, among other things, the generality of the disease and its significance as a cause of (a) mortality (b) inability to work, (c) economic loss, and (d) suffering [2] Although anaemia is common, excess mortality because of it is insignificant and mainly due to severe diseases which have caused the anaemia [12, 25 29] In addition low haemoglobin values have not been found to influence submaximal physical performance [5] although the maximal performance of anaemic persons has been found to decrease [31] Therefore, slight anaemias have not been shown so far at any rate, to present any substantial public health problem as a cause of inability to work and economic loss. Anaemia is not a significant cause of suffering either Investigation results on the correlation between symptoms and the level of haemoglobin are contradictory in that some investigators [16] reported a correlation between them while others [10 32] found no increase in symptoms as the haemoglobin level decreased, at least not in mild or moderately severe anaemias.

If the haematocrit values of HCT $< 35\%$ and HCT $< 40\%$ for women and men respectively are regarded as criteria for anae

30 Wilson, J. M. G. and Jungner, G.: Principles and practice of screening for disease. Public Health Papers 34 (WHO, Geneva 1968).
31 Viteri, F. E. and Torún, B.: Anaemia and physical work capacity. Clin. Haematol. *3*: 609–626 (1974).
32 Wood, M. M. and Elwood, P. C.: Symptoms of iron deficiency anaemia. A community survey. Br. J. prev. soc. Med. *20*: 117–121 (1966).
33 WHO: Nutritional anaemias. Report of a WHO group of experts. WHO Tech. Rep. Ser. No. 503 (WHO, Geneva 1972).

Received: January 30, 1978
Accepted: July 1, 1978

Dr. Jorma Takala, Saarijärventie 10 A 1, SF–40200 Jyväskylä 20 (Finland)

3 Beveridge, B. R. Bannerman, R. M Evonson, J M and Witts, L J Hypochromic anaemia. A retrospective study and follow-up of 378 in-patients. Q Jl Med. *34* 145–161 (1965)

4 Brunning, R. D Differential diagnosis of anaemia. Geriatrics *29* 52–60 (1974).

5 Cotes, J E. Dabbs, J M. Elwood, P C. Hall, A. M McDonald, A., and Saunders, M J.. The response to submaximal exercise in adult females, relation to haemoglobin concentration. Proc. Physiol. Soc. J *1969* 79–80.

6 Dagg, J H Blood and neoplastic diseases. Treatment of iron deficiency and iron over load Br med. J *II* 494–497 (1974)

7 *Davis, R. E., Kelsall, G R. H., Stenhouse N* S. Woodliff H. J and Wearne, J T.. Haemoglobin and haematocrit measurements in a community Report from the Busselton survey 1966. Med. J Aust. *II.* 1196–1200 (1969).

8 De Gruchy G C.. Clinical haematology in medical practice (Blackwell Scientific Publications, Oxford 1973).

9 Elwood, P C., Waters, W E. Greene W J and Wood, M M Evaluation of a screening survey for anaemia in adult non-pregnant women. Br med. J *II* 714–717 (1967).

10 Elwood, P C., Waters, W E., Greene, W J W Sweetnam, P., and Wood, M. M Symptoms and circulating haemoglobin level J chron. Dis. *21* 615–628 (1969).

11 Elwood, P C.. The clinical evaluation of circulating haemoglobin level. Clin. Haematol. *3* 705–718 (1974).

12 Elwood, P C. Benjamin, I. T Waters, W E., and Sweetnam, P M Mortality and anaemia in women. Lancet *II* 891–894 (1974)

13 Elwood, P C. Anaemia. Lancet *II.* 1364–1365 (1974)

14 French, D G Discussion on anaemia in general practice Proc R. Soc. Med. *45* 347 354 (1955).

15 Fry J Clinical patterns and course of anaemias in general practice. Br med. J II 1732 1736 (1961).

16 Hjelm, M. and Wadman, B. Clinical symptoms, haemoglobin concentration and erythrocyte biochemistry A study in women with iron deficiency anaemia. Clin. Haematol. *3* 689–703 (1974).

17 Kilpatrick, G S. and Hardisty R. M.. The prevalence of anaemia in the community A survey of a random sample of the population. Br med. J *I* 778–782 (1961)

18 Kilpatrick, G S.. Prevalence of anaemia in the general population. A rural and an industrial area compared. *Br med. J II.* 1736–1738 (1961).

19 Kilpatrick, G S.. Anaemia. The presymptomatic diagnosis of anaemia. Proc. R. Soc. Med. *59* 1220–122.. (1966).

20 Linman, J W.. Differential diagnosis of hypochromic anaemias. Postgrad. Med. *49* 88–94 (1971).

21 Mean blood haematocrit of adults, United States, 1960–196... National Center for Health Statistics series 11 No. 24 (US Department of Health, Education, and Welfare, Public Health Service, Washington 1967)

22 Natvig, H. and Vellar O D Studies on hemoglobin values in Norway VIII Hemoglobin, Hematocrit and MCHC values in adult men and women. Acta med. scand. *182* 193–205 (1967)

23 Socialstyrelsen Hälsoundersökningen i Värmland 1962–1965 (Civiltryck, Stockholm 1968).

24 Takala, J Sievers, K., and Takala, A.. A multiphasic screening programme at the health centre level the Säkylä-Köyliö project. The variables, methods and participation. Scand. J soc. Med. (in press)

25 Takkunen, H and Aromaa, A.. Mortality and anaemia. Lancet *II* 523–524 (1974)

26 Takkunen, H and Seppänen R.. Iron deficiency and dietary factors in Finland. Am. J clin. Nutr *28* 1141–1147 (1975).

27 Takkunen, H Iron deficiency in the Finnish adult population. An epidemiological survey from 1967 to 1972 inclusive. Scand. J Haematol., suppl 25 (Munksgaard, Copenhagen 1976).

28 Walker A. R. P.. Controversy on iron needs, intake levels, deficiency stigmata and benefits from iron supplementation. Post-grad. med. J *45* 747–753 (1969)

29 Waters, W E., Withey J L. Kilpatrick, G S., Wood, P H N and Abernethy M.L. Ten-year haematological follow-up mortality and haematological changes. Br med. J *I* 761 764 (1969).

30 Wilson, J M. G and Jungner G Pr and practice of screening for disease Health Papers 34 (WHO Geneva 1968
31 Viteri, F E. and Torún, B Anaemia physical work capacity Clin. Haemat. 609–626 (1974).
32 Wood, M. M. and Elwood, P C. Symp of iron deficiency anaemia A community sur vey Br J prev soc. Med. 20 117 121 (1966)
33 WHO Nutritional anaemias. Report of

Dr Jorma Takala, Seu ,ar oatie 10 A 1
52 40.00 Jyvaskyla 2 (Finland)

Acta haemat. 61 114–119 (1979)

Haemoglobin Barts in Newborn Tanzanians

A M Nhonoli J M Kujwalile P W Mmari and Y Shemaghoda
Muhimbili Medical Centre, Faculty of Medicine, University of Dar es Salaam, Dar es Salaam

Key Words. Haemoglobin Barts Newborn Tanzanians α Thalassaemia Electrophoresis β γ-Chains

Abstract. Using both starch gel and cellulose acetate electrophoresis as screening procedures, haemoglobin (Hb) Barts was detected in 11.08% of 325 cord blood samples from newborn Tanzanians. Red cell studies in these and in normals and a search for inclusion bodies of Hb H did not suggest α-thalassaemia. The mothers of these babies do not show any evidence of α-thalassaemia. It is suggested that the presence of Hb Barts in samples of cord blood is not due to the presence of α-thalassaemia in the Tanzanian population

Introduction

Haemoglobin (Hb) Barts was first described by *Ager and Lehmann* [1] as a rapidly migrating component At birth, Hb F ($\alpha_2\ \gamma_2$) forms about 50% of the Hb mass during the perinatal period, Hb A ($\alpha_2\ \beta_2$) replaces Hb F as the main Hb This process proceeds smoothly without imbalance of globin chain production but traces of Hb Barts (γ4) are found in most samples of cord blood. This may indicate a minor degree of chain imbalance during this switch-over process. However *Weatherall* [15] *Wasi et al.* [13] *Lehmann* [7] *Pembrey et al* [10] claim that the presence of increased amounts of Hb Barts in the neonatal period is the most sensitive indicator of the presence of α-thalassaemia gene On the other hand *Esan* [4 5] has suggested that increased amounts of Hb Barts do not always indicate the presence of α-thalassaemia and may represent a development abnormality that is asynchronous of the neonatal switch off of γ-chains and activation of β-chain production. In thalassaemia there is globin-chain imbalance, low MCH abnormal red cell morphology especially anisocytosis, poikilocytosis and target cell preponderance *Esan* [4 5] found that osmotic fragility haematological values of these with Hb Barts compared with babies without this Hb did not suggest α-thalassaemia The parents of these babies showed no evidence of α-thalassaemia and had no inclusion bodies of Hb H *Pembrey et al* [10] have shown that normal Caucasian cord blood contains quantities of Hb Barts which are less than

0.5 /o, and they consider any higher levels as being abnormal

Materials and Methods

Samples of cord blood were collected consecutively at the time of delivery t the Maternity Block between May 1975 and April 1976, 325 such samples were collected. Blood from mothers f infants shown to have Hb Barts was collected Fathers' blood could not be obtained as it was impracticable to obtain samples from them.

The cord blood was collected in heparinized sterile universal bottles Samples were immediately run through the Coulter Counter Model S. Thin blood films were made for differential counts, and for red cell morphology These were stained with May-Grunwald-Giemsa stain After the first 100 consecutive bloods were done, it was decided to put through the Coulter only bloods with Hb Barts and that of thei mothers

Hb Electrophoresis

Haemolysates were prepared as described by *Lehmann and Huntsman* [8] and the final concentration of the haemolysate was adjusted to 8 g dl with distilled water Electrophoresis was performed by both cellulose-acetate strips and horizontal starch-gel methods using the Tris-EDTA borate system at pH 8 6 and stained with amido black after *electrophoresis in starch gel, as described by Weatherall and Clegg* [16]. Samples which were found to have fast-moving band were further subjected to horizontal starch-gel electrophoresis using phosphate buffer pH 6.5, for confirmation of *Hb Barts* and then stained with amido black. I each case, 5 µl of the *haemolysate are applied Samples from mothers of infants* with Hb Barts were electrophoretically screened in the same way for Hb H

Quantitation f Hb Barts

This was done by cellulose-acetate electrophoresis using the Tris-EDTA-borat system at pH 8 6 as described by *West and Pootrakul* [14] Samples with known concentrations of Hb Barts re included as controls Every value obtained is the verage *of duplicate determinations which did not* differ by more than 0.4% , whenever they did the quantitation was repeated.

Those samples which had less than 4% Hb Barts were quantitated after horizontal starch-gel *electrophoresis in phosphate buffer* at pH 6.5. Briefly following electrophoresis, excess buffer was removed from the block using filter paper at each end, and then sectioning of the gel into tw slices of equal thickness. One of the slices was placed on smooth bench surface and Hb frac tions visualized over strong light, then cut out, *placed in sintered glass funnels, allowed to stand* for 30 min in phosphate buffer at 4 °C. They were then eluted into graduated tubes. Dilutions were then made, the Hb A, Hb F etc. mixture was di-



Fig. 1. Starch-gel electrophoresis (Tris-EDTA-borate system, pH 8.6, amido black stain) of tw cord blood *samples containing Hb Barts.*

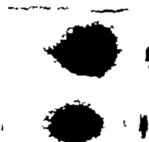


Fig. 2. Starch-gel electrophoresis (phosphate buffer system, pH 6 5 amido black stain). The anodal migration of Hb Barts distinguishes it from the A+F mixture which migrates towards the cathode.

Acta haemat 61 114–119 (1979)

Haemoglobin Barts in Newborn Tanzanians

A M Nhonoli J M Kupwalile P B Mmari and Y Shemaghoda

Muhimbili Medical Centre Faculty of Medicine, University of Dar es Salaam, Dar es Salaam

Key Words. Haemoglobin Barts Newborn Tanzanians α-Thalassaemia Electrophoresis β- γ-Chains

Abstract. Using both starch gel and cellulose-acetate electrophoresis as screening procedures haemoglobin (Hb) Barts was detected in 11 08% of 325 cord blood samples from newborn Tanzanians. Red cell studies in these and in normals and a search for inclusion bodies of Hb H did not suggest α-thalassaemia The mothers of these babies do not show any evidence of α-thalassaemia. It is suggested that the presence of Hb Barts in samples of cord blood is not due to the presence of α-thalassaemia in the Tanzanian population

Introduction

Haemoglobin (Hb) Barts was first described by *Ager and Lehmann* [1] as a rapidly migrating component At birth, Hb F (α_2 γ_2) forms about 50% of the Hb mass, during the perinatal period, Hb A (α_2 β_2) replaces Hb F as the main Hb This process proceeds smoothly without imbalance of globin chain production but traces of Hb Barts (γ4) are found in most samples of cord blood. This may indicate a minor degree of chain imbalance during this switch over process However *Weatherall* [15] *Wasi et al* [13] *Lehmann* [7] *Pembrey et al* [10] claim that the presence of increased amounts of Hb Barts in the neonatal period is the most sensitive indicator of the presence of α-thalassaemia gene On the other hand, *Esan* [4 5] has suggested that increased amounts of Hb Barts do not always indicate the presence of α-thalassaemia and may represent a development abnormality that is asynchronous of the neonatal switch off of γ-chains and activation of β-chain production In thalassaemia there is globin-chain imbalance low MCH abnormal red cell morphology especially anisocytosis, poikilocytosis and target cell preponderance *Esan* [4 5] found that osmotic fragility haematological values of these with Hb Barts compared with babies without this Hb did not suggest α-thalassaemia. The parents of these babies showed no evidence of α-thalassaemia and had no inclusion bodies of Hb H *Pembrey et al* [10] have shown that normal Caucasian cord blood contains quantities of Hb Barts which are less than

0.5 /o, and they consider any higher levels as being abnormal

Materials and Methods

Samples of cord blood were collected consecutively at the time of delivery at the Maternity Block between May 1975 and April 1976, 325 such samples were collected Blood from mothers of infants shown to have Hb Barts was collected Fathers blood could not be obtained as it was impracticable to obtain samples from them.

The cord blood was collected in heparinized sterile universal bottles. Samples were immediately run through the Coulter Counter Model S Thin blood films were made for differential counts, and for red cell morphology These were stained with May-Grunwald-Giemsa stain. After the first 100 consecutive bloods were done, it was decided to put through the Coulter only bloods with Hb Barts and that of their mothers

Hb Electrophoresis

Haemolysates were prepared as described by *Lehmann and Huntsman* [8] nd the f nal concentration of the haemolysate was adjusted t 8 g dl with distilled water Electrophoresis was performed by both cellulose-acetate strips and horizontal starch-gel methods using the Tris-EDTA-borat system at pH 8 6 and stained with amido black fter electrophoresis in starch gel, as described by *Weatherall and Clegg* [16]. Samples which were found to have fast-moving band were further subjected to horizontal starch-gel electrophoresis using phosphat buffer pH 6.5 for confirmation of Hb Barts and then stained with amido black. I each case, 5 1 of the haemolysate are applied. Samples from mothers of infants with Hb Barts were lectrophoretically screened in the same way for Hb H

Quantitation J Hb Bart

This was done by cellulose-acetate electrophoresis using the Tris-EDTA-borate system at pH 8 6 as described by *Wast and Poetrakal* [14] Samples with know concentrations of Hb Barts were included as controls. Every value obtained is the average of duplicate determinations which did not differ by more than 0 4% a, whenever they did the quantitation was repeated.

Those samples which had less than 4% Hb Barts were quantitated after horizontal starch-gel electrophoresis in phosphate buffer at pH 6.5 Briefly following electrophoresis, excess buffer was removed from the block using filter paper at each end, and then sectioning of the gel into tw slices of equal thickness. One of the slices was placed on smooth bench surface and Hb fractions visualized over strong light, then cut out, placed in sintered glass funnels, allowed to stand for 30 min in phosphate buffer at 4 °C. They were then eluted into graduated tubes. Dilutions were then made, the Hb A, Hb F etc. mixture was di-



Fig. 1. Starch-gel electrophoresis (Tris-EDTA-borate system, pH 8 6, amido black stain) of two cord blood samples containing Hb Barts



Fig. 2. Starch-gel electrophoresis (phosphate buffer system, pH 6 5 amido black stai) The anodal migration of Hb Barts distinguishes it from the A+F mixture hich migrates towards the cathode.

luted to 15 times the volume of Hb Barts. Eluates were then cleared of particulate matter by means of centrifugation at 3,000 R.p.m. for 5 min and optical densities read in the Eppendorf spectrophotometer at a wavelength of 546 nm A gel free of Hb was treated in the same way and the filtrate used as a blank. In each case, duplicate determinations were done and the values obtained did not differ by more than 0.3%.

Quantiation of Hb F

This was performed by the 1 min denaturation method of *Singer et al* [11].

Hb H Inclusion Bodies

This test was performed on the blood of mothers of those infants showing Hb Barts. 2 vol of fresh blood and 1 vol of 1 % brilliant cresyl blue in 0.9% sodium chloride were mixed and incubated at 37 C for at least 1 h. Dry blood films were then prepared at intervals of 1 and ½ h, and each slide examined under oil immersion for a minimum of 4 min before being ruled out to be negative for Hb H inclusion bodies. Only one batch of brilliant cresyl blue was used throughout. Although we did not have a positive control for Hb H inclusion bodies the test system according to our experience produced reliable results whenever used for the reticulocyte counts.

Red Cell Osmotic Fragility

This was performed on mothers of infants with Hb Barts only by the method based upon that of *Parpart et al* as described by *Dacie and Lewis* [3]. It was not done on cord bloods because it is known that the range in cord bloods is very wide [9] with a minimal distribution, hence separation of normal from abnormal is arbitrary and the test does not differentiate between the two groups. Secondly *Esan* [5] showed a very poor correlation between the median corpuscular fragility and the concentration of Hb Barts in the blood.

Results

37 of the cord bloods showed presence of Hb Barts (i e 11 08%) in quantities ranging from 0 9 to 10 7%

Our incidence of 37 cases out of 325 cord bloods (11 08%) compares well with that reported from other parts of Africa. *Esan* [5] in Nigerians got 5 1% out of 1 866 cord bloods *Hendrickse et al.* [6] in Nigerians got 10% out of 140 cord bloods. *Van Baelen et al* [12] in the then Congo (now Zaire) got 17 9% out of 636 cord bloods. The highest rate has been reported outside Africa in Shute Saudi Arabian cord bloods by *Pembrey et al* [10] who found a rate of 52% out of 345 cord bloods.

Red Cell Indices on Blood Smears

Normal non-Hb Barts cord bloods showed normochromasia with slight anisocytosis and poikilocytosis. Those with Hb Barts were all normochromic, and showed moderate anisocytosis and poikilocytosis, with slight diffuse polychromasia with occasional target cells. These changes were minimal and could not be construed as constituting a possibility of the presence of α-thalassaemia.

No Evidence of α Thalassaemia in Mothers

The mothers blood showed a normal morphology Hb electrophoresis showed no Hb Barts and no Hb H bands Hb H inclusion bodies were not found in any of these mothers. The osmotic fragility in these mothers was normal in all cases.

Comparison of Red Cell Indices

Table I shows these indices in 33 babies with Hb Barts, 20 babies with levels of Hb Barts less than 5 0%, and 80 normal babies. 4 babies with Hb Barts were left out because some of the red cell indices were incomplete only 80 of the 90 normal bloods had complete records. Comparing the 80

Table I. Comparison of red cell indices in the normal and Hb Barts babies (mean ± SEM)

	33 Hb Barts babies	20 Hb Barts babies with Hb Barts < 5.0%	80 normal babies	Normal compared to Hb Barts
PCV %	44.33 ± 0.519	43.25	46.73 ± 0.346	$p<0.05$
MCH pg	29.14 ± 0.441	29.63	32.43 ± 0.334	$p<0.05$
MCHC, g/dl	30.40 ± 0.555	30.78	31.11 ± 0.184	p 0.05
MCV fl	96.33 ± 0.455	97.20	104.85 ± 0.336	$p<0.05$
Hb F	72.55 ± 0.911	71.58	71.8 ± 0.297	p 0.05

normal babies with the 33 abnormal babies it is shown that babies with Hb Barts have a significantly lower haematocrit, mean corpuscular Hb and mean corpuscular volume than the normal babies. However the mean corpuscular Hb concentration seems not to be affected by the presence of Hb Barts.

Hb F in babies with Hb Barts shows mean levels of 72.55%, whilst normal babies have a mean of 71.8%, the difference is not statistically significant.

Statistical Relationship between Hb Barts and Red Cell Indices in Babies with Hb Barts Levels Less than 5 %

The 20 babies with levels of less than 5.0% Hb Barts were put in a group of their own because the scattergrams in this group with respect to the red cell indices showed a linear correlation on inspection. This group was subjected to a detailed statistical analysis. The statistical methods used are explained in *Croxton et al.* [2]. It should be noted that.

(i) *The coefficient of gross correlation (r)* shows that of the three relationships to Hb Barts the MCV (–0.647864) shows the highest gross negative correlation with Hb Barts, followed by that of MCH (–0.5749) and least by that of MCHC (–0.47842).

(ii) *The coefficients of partial correlation (r)* show that the closeness of relationship *between Hb Barts and* (a) *MCV* when MCHC is held constant (r143) is –0.53026, and when MCH is held constant (r142) is –0.36936 (b) *MHC* when MCHC is held constant (r123) is –0.507430 and when MCV is held constant (r124) is +0.0604259 (c) *MCHC* when MCH is held constant (r132) is –0.38072, and when MCV is held constant (r134) is –0.2116069.

This again shows that the negative relationship is highest between Hb Barts and MCV followed by that between Hb Barts and MCH and least between Hb Barts and MCHC.

(iii) *The coefficient of multiple determination (R^2...)* shows that: (a) In R 124 we have explained 42.18% of the variation in Hb Barts. (b) In R 123 we have explained 42.74 % of this variation. (c) In R 134 we have explained 44.57% of this variation. (d) In R 1234 that is, when the effect of all three dependent variables are combined we have explained only 44.7% of the variation in Hb Barts.

This shows that there are more other factors acting in explaining the Hb Barts variation, than the three dependent variables that we have investigated, as these ac

count only for just under half of the total variation

(iv) Taking the relationship between MCH and MCV the coefficient of gross correlation (r_{21}) is + 0 9159 This would seem to show that there is a close direct correlation between these two variables in babies with Hb Barts However in the 80 normal babies (last column in table I) the coefficient of gross correlation (r_{21}) is + 0 84060 Thus this direct close relationship between MCH and MCV is not specific in those with Hb Barts, but it is a natural relationship which holds true in normals

Comment

Hb Barts occurs in about 11% of Tanzanian babies at birth in amounts up to 11% of the Hb mass Hb H disease has not been seen in this country up to now and no single case of hydrops foetalis has been demonstrated to be connected with Hb Barts in this country up to the present The mothers of these babies with Hb Barts have been shown to have a normal red cell morphology normal osmotic fragility and to show no inclusion bodies. We agree with *Esan* [5] that Hb Barts in the African newborn tested so far seems not to be related to α-thalassaemia It is probably a relative excess of γ-polypeptide chains of Hb resulting either from a decrease in α-chain production or an increase in γ-chain synthesis during the switch-over mechanism to β-chains at about the time of birth We have no facilities for biosynthesis studies. These comments must be taken bearing this in mind Attempts are being made to trace these children to see whether or not their Hb Barts has disappeared.

Statistical analysis in those with amounts of Hb Barts less than 5% of the Hb mass shows that this Hb Barts is statistically negatively related to MCV MCH and MCHC in that descending order However this does not explain all the variations and there must be other factors which are related to Hb Barts.

References

1 Ager J A M and Lehmann, H Observations on some 'fast' haemoglobins, K, J N and Barts Br med. J *I* 929–931 (1958).
2 Croxton, F E Cowden, D J and Klein, S Applied general statistics 3rd ed. (Pitman, London 1968)
3 Dacie, J V and Lewis, S. M. Osmotic fragility Practical haematology 5th ed., 202 208 (Churchill/Livingstone Edinburgh 1975).
4 Esan, G J F The thalassaemia syndromes in Nigeria. Br J Haemat. *19* 47–56 (1970).
5 Esan, G J F Haemoglobin Bart s in in newborn Nigerians. Br J Haemat. *22* 73–86 (1972).
6 Hendrickse R. G Boyo, A. E. Fitzgerald, P A., and Ransome Kuti, S.. Studies on the Haemoglobins of newborn nigerians. Br med. J *I* 611–614 (1960)
7 Lehmann, H. Different types of alpha-thalassaemia and significance of haemoglobin Bart s in neonates. Lancet *II* 78–79 (1970).
8 Lehmann, H and Huntsman, R. G Preparation of haemoglobin solution. Man s haemoglobins, including the haemoglobinopathies and their investigation 2nd ed pp 393–394 (North Holland, Amsterdam 1974).
9 Luzzatto, L. Esan, G J F and Ogiemudia, S E. The osmotic fragility of red cells in newborns and infants. Acta haemat *43* 248–256 (1970)
10 Pembrey M E. Weatherall D J Clegg, J B. Bunch, C., and Perrine, R. P Haemoglobin Bart s in Saudi Arabia. Br J Haemat. 29 221–233 (1975)
11 Singer K. Chernoff A. L, and Singer L. Studies on abnormal haemoglobins. I Their

demonstration in Sickle cell anaemia and other haematological disorders by means of alkali denaturation. Blood 6 413–428 (1951).
12 Van Baelen, H Vandepitte, J Cornu, G., and Eeckels, R. Routine detection of sickle cell anaemic and haemoglobin Bart' in Congolese neonates. Trop. geogr Med. 21 41.–426 (1969).
13 Wasi, P Na-Nakorn, S., Pootrakul, S., Sookanek, M. Disthasongchan, P Pornpatkul, M., and Panich, V Alpha and beta-thalassaemia in Thailand. Ann. N.Y Acad. Sci. 165 60–82 (1969).
14 Wasi, P and Pootrakul, S Laboratory diagnosis of alpha-thalassaemia. The detection of haemoglobinopathies, p 91 (CRC Press, Ohio 1974)
15 Weatherall, D J Abnormal haemoglobins in the neonatal period and their relationship to thalassaemia. B J Haemat. 9 265–277 (1963).
16 Weatherall, D. J and Clegg, J B. Starch gel electrophoresis. The thalassaemia syndromes; 2nd ed. pp. 308–310 (Blackwell Scientific Publications, Oxford 1972).

Received. May 31, 1977
Accepted: May 25, 1978

A. M. Nhonoli,
Muhimbili Medical Centre, Faculty of Medicine
University f Dar es Salaam,
Dar es Salaam (Tanzania)

count only for just under half of the total variation.

(iv) Taking the relationship between MCH and MCV the coefficient of gross correlation (r_{24}) is + 0 9159 This would seem to show that there is a close direct correlation between these two variables in babies with Hb Barts However in the 80 normal babies (last column in table I) the coefficient of gross correlation (r_{24}) is + 0 84060 Thus this direct close relationship between MCH and MCV is not specific in those with Hb Barts, but it is a natural relationship which holds true in normals.

Comment

Hb Barts occurs in about 11% of Tanzanian babies at birth in amounts up to 11% of the Hb mass Hb H disease has not been seen in this country up to now and no single case of hydrops foetalis has been demonstrated to be connected with Hb Barts in this country up to the present. The mothers of these babies with Hb Barts have been shown to have a normal red cell morphology normal osmotic fragility and to show no inclusion bodies. We agree with *Esan* [5] that Hb Barts in the African new born tested so far seems not to be related to α-thalassaemia It is probably a relative excess of γ-polypeptide chains of Hb resulting either from a decrease in α-chain production or an increase in γ-chain synthesis during the switch-over mechanism to β-chains at about the time of birth We have no facilities for biosynthesis studies These comments must be taken bearing this in mind. Attempts are being made to trace these children to see whether or not their Hb Barts has disappeared

Statistical analysis in those with amoun of Hb Barts less than 5% of the Hb ma shows that this Hb Barts is statistically ne atively related to MCV MCH and MCH in that descending order However th does not explain all the variations and ther must be other factors which are related t Hb Barts.

References

1 Ager J A M and Lehmann, H. Observatio on some 'fast' haemoglobins, K J N, an Barts Br med. J *1* 929–931 (1958).

2 Croxton, F E Cowden, D J and Klein, S Applied general statistics, 3rd ed. (Pitma London 1968)

3 Dacie, J V and Lewis, S. M Osmotic fragil ty Practical haematology 5th ed., 202 (Churchill/Livingstone, Edinburgh 1975)

4 Esan, G J F. The thalassaemia syndromes i Nigeria. Br J Haemat. *19* 47–56 (1970).

5 Esan, G J F Haemoglobin Bart s in in nev born Nigerians. Br J Haemat. 2 73– (1972)

6 Hendrickse, R. G Boyo, A. E. Fitzgerald, A., and Ransome Kuti, S. Stud es on the Ha moglobins of newborn nigerians. Br med. J 611–614 (1960).

7 Lehmann, H Different types of alpha-thala ssemia and significance of haemoglobin Bart in neonates. Lancet *II* 78–79 (1970)

8 Lehmann H and Huntsman, R G Prepar tion of haemoglobin solution. Man s haem globins, including the haemoglobinopathi and their investigation 2nd ed pp 393–3 (North Holland, Amsterdam 1974)

9 Luzzatto, L. Esan G J F and Ogiemudia, E. The osmotic fragility of red cells in nev borns and infants. Acta haemat *43* 248–25 (1970).

10 Pembrey M E. Weatherall D J Clegg, B. Bunch, C., and Perrine, R. P Haemogl bin Bart s in Saudi Arabia. B J Haemat. 2 221–233 (1975)

11 Singer K. Chernoff A. I and Singer Studies on abnormal haemoglobins. I The

Original Papers

Acta haemat. *61* 121 124 (1979)

Hemoglobin Moabit Alpha 86 (F7) Leu → Arg

A New Unstable Abnormal Hemoglobin

A Knuth W Pribilla, H R Marti and K. H Winterhalter

Krankenhaus Moabit, II. Innere Abteilung, Berlin,
Kantonsspital Aarau, Department of Internal Medicine, Aarau;
Friedrich Miescher-Institut, CIBA AG Basel

Key Words. Unstable abnormal hemoglobin Congenital Heinz body hemolytic anemia

Abstract. A new alpha chain abnormal hemoglobin variant was found in a Turkish patient with a mild Heinz body hemolytic anemia and splenomegaly The substitution alpha 86 Leu → Arg, which is next to the heme binding proximal histidine is responsible for a marked instability of the molecule The oxygen affinity of the erythrocytes was found to be slightly decreased.

Among approximately 300 known abnormal hemoglobins, more than 50 are unstable The instability of most of these variants can be attributed to an amino acid substitution in the vicinity of the heme pocket or in the interior of the folded poly peptide chain. In this paper a new unstable abnormal hemoglobin due to the substitution of an amino acid participating in the heme contact and the resulting clinical disorder will be described.

Material and Methods

Hemolysates were prepared from washed erythrocytes using CCl_4. Electrophoresis was performed on starch block with barbital buffer pH 8 6 Hb F was determined by *Betke* method of alkali denaturation [1] The instability of the variant was detected by means of the isopropanol test [4]. For the heat stability test solution containing about 1 g Hb/dl in Tris buffer pH 7 4 0 1 *M* was incubated 5 min at 65 °C. The Heinz body formation was tested in erythrocytes incubated with 0 045 *M* $NaNO_2$, 0.07 *M* glucose and 0 0001 *M* methylene blue at 37 °C for 3 h. The abnormal polypeptide chains were identified using electrophoresis on cellogel strips [6]. The methods used for the preparation of globin, its separation into alpha and beta chains by chromatography the fingerprinting of tryptic digests, and the amino acid analysis of the abnormal peptide have recently been described elsewhere [8]. For the determination of red cell enzymes the methods indicated by *Beutler* [2] were used with slight modifications. The oxygen affinity of red cells was estimated by determination of P_{50} [7]

Results

Clinical Case Report

A 38-year-old patient from Turkey was admitted to the hospital because of a spleno-

Book Reviews

R. Biggs (ed.)
The Treatment of Haemophilia A and B and von Willebrand's Disease
Blackwell Oxford 1978
XII + 242 pp £ 12.00
ISBN 0-632-00216-6

According to the editor's preface, this monograph on haemophilia and related disorders has been deliberately biased to reflect experience gained at the Oxford Haemophilia Centre. Various chapters on the physiology and biochemistry of haemostasis, laboratory evaluation of blood dyscrasias, economical aspects of blood component therapy minimal (and optimal) treatment of haemophiliacs, home therapy complication of substitution therapy and organisation of haemophilia treatment have all been written by former or present members of the Oxford Haemophilia Centre. Thus, the text is unusually cohesive.

In reading this book, arguments of one kind or another come to mind. However objections become very subjective indeed when one enters the wealth of clinical experience contained in this book. In fact, the work of the editor and her coworkers represents an outstanding example of continuing retrieval of medico-social and scientific data performed by a relatively small group of devoted physicians and biochemists. The general presentation is outstanding, and a few misspelled names (Favre-Gilly p 28 Simonetti, p. 35) are forgiveable.

In a final chapter some rather philosophical – if not political – thoughts about medical specialisation are being presented It seems to us that there may be a general lesson to be learnt from our study of haemophiliacs. This lesson i in the simple thought that more attention should be given to the care of individual patients with individual diseases and less to overall hospital organisation (p 233)

I hope that with these few remarks I have conveyed the idea that this is an unusual text book and expressed some of the pleasure which I experienced in reading this monograph.

E. A. Beck, Bern

W J Rudowski (ed.)
Disorders of Hemostasis in Surgery
The University Press of New England,
Hanover New Hampshire 1977
456 pp US $ 17.50
ISBN 0-87451-137-2

This book has been written by a group of Polish physicians and biochemists and was translated under a joint program with the US National Library of Medicine. The goal set specifically by the editor of this book, i.e., 'making surgery safer for the patient, and the patient a safer object of surgery' is commendable. The title is, however somewhat misleading: about two thirds of the book deal with theoretical aspects, whereas problems affecting the surgeon more directly are hidden behind such titles as 'hemostatic side effects of drugs Crucial problems, such as preoperative screening programs and 'pharmacologic hemostasis are treated rather superficially

The book is inexpensive and contains some useful information. It still leaves space open for a truly interdisciplinary text book dealing with disorders of hemostasis in surgery'

E. A. Beck, Bern

Original Papers

Acta haemat. 61: 121-124 (1979)

Hemoglobin Moabit: Alpha 86 (F7) Leu → Arg

A New Unstable Abnormal Hemoglobin

A. Knuth, W. Pribilla, H. R. Marti and K. H. Winterhalter

Krankenhaus Moabit, II. Innere Abteilung, Berlin;
Kantonsspital Aarau, Department of Internal Medicine, Aarau
Friedrich Miescher Institut, CIBA AG, Basel

Key Words. Unstable abnormal hemoglobin · Congenital Heinz body hemolytic anemia

Abstract. A new alpha chain abnormal hemoglobin variant was found in a Turkish patient with a mild Heinz body hemolytic anemia and splenomegaly. The substitution alpha 86 Leu → Arg, which is next to the heme binding proximal histidine, is responsible for a marked instability of the molecule. The oxygen affinity of the erythrocytes was found to be slightly decreased.

Among approximately 300 known abnormal hemoglobins, more than 50 are unstable. The instability of most of these variants can be attributed to an amino acid substitution in the vicinity of the heme pocket or in the interior of the folded polypeptide chain. In this paper a new unstable abnormal hemoglobin due to the substitution of an amino acid participating in the heme contact and the resulting clinical disorder will be described.

Material and Methods

Hemolysates were prepared from washed erythrocytes using CCl_4. Electrophoresis was performed on starch block with barbital buffer pH 8.6. Hb F was determined by *Betke's* method of alkali denaturation [1]. The instability of the variant was detected by means of the isopropanol test [4]. For the heat stability test, a solution containing about 1 g Hb/dl in Tris buffer pH 7.4 0.1 *M* was incubated 5 min at 65 °C. The Heinz body formation was tested in erythrocytes incubated with 0.045 *M* $NaNO_2$, 0.07 *M* glucose and 0.0001 *M* methylene blue at 37 °C for 3 h. The abnormal polypeptide chains were identified using electrophoresis on cellogel strips [6]. The methods used for the preparation of globin, its separation into alpha and beta chains by chromatography, the fingerprinting of tryptic digests, and the amino acid analysis of the abnormal peptide have recently been described elsewhere [8]. For the determination of red cell enzymes the methods indicated by *Beutler* [2] were used with slight modifications. The oxygen affinity of red cells was estimated by determination of P_{50} [7].

Results

Clinical Case Report

A 38-year-old patient from Turkey was admitted to the hospital because of a spleno-

Book Reviews

R Biggs (ed.)
The Treatment of Haemophilia A and B and von Willebrand's Disease
Blackwell, Oxford 1978
XII + 242 pp., £ 12.00
ISBN 0-632-00216-6

According to the editor's preface, this monograph on haemophilia and related disorders has been 'deliberately biased' to reflect experience gained at the Oxford Haemophilia Centre. Various chapters on the physiology and biochemistry of haemostasis, laboratory evaluation of blood dyscrasias, economical aspects of blood component therapy, minimal (and optimal) treatment of haemophiliacs, home therapy, complication of substitution therapy and organisation of haemophilia treatment have all been written by former or present members of the Oxford Haemophilia Centre. Thus, the text is unusually cohesive.

In reading this book, arguments of one kind or another come to mind. However, objections become very subjective indeed when one enters the wealth of clinical experience contained in this book. In fact, the work of the editor and her coworkers represents an outstanding example of continuing *retrieval of medico-social and scientific data, per*formed by a relatively small group of devoted physicians and biochemists. The general presentation is outstanding, and a few misspelled names (Favre-Gilly, p 28, Simonetti, p 35) are forgiveable.

In a final chapter, some rather philosophical – if not political – thoughts about medical specialisation are being presented: 'It seems to us that there may be a general lesson to be learnt from our study of haemophiliacs. This lesson is in the simple thought that more attention should be given to the care of individual patients with individual diseases and less to overall hospital organisation' (p 233).

I hope that with these few remarks I have conveyed the idea that this is an unusual text book and expressed some of the pleasure which I experienced in reading this monograph.

E. A. Beck, Bern

W J Rudowski (ed.)
Disorders of Hemostasis in Surgery
The University Press of New England,
Hanover, New Hampshire 1977
456 pp., US $ 17.50
ISBN 0-87451-137-2

This book has been written by a group of Polish physicians and biochemists and was translated under a joint program with the US National Library of Medicine. The goal set specifically by the editor of this book, i.e., 'making surgery safer for the patient, and the patient a safer object of surgery' is commendable. The title is, however, somewhat misleading: about two thirds of the book deal with theoretical aspects, whereas problems affecting the surgeon more directly are hidden behind such titles as 'hemostatic side effects of drugs'. Crucial problems, such as preoperative screening programs and 'pharmacologic hemostasis' are treated rather superficially.

The book is inexpensive and contains some useful information. It still leaves space open for a truly interdisciplinary text book dealing with disorders of hemostasis in surgery.

E. A. Beck, Bern

Fig. 2. Starch block electrophoresis: right = propositus; left = control.

Fig. 3. Primary structure of the heme binding part of the alpha polypeptide chain (arrow indicate the amino acid substitution to Hb Moabit)

stead of 0.2–5 / in normal hemolysates. It can be concluded that the variant represents approximately 15 / of the total hemoglobin present in the hemolysate. The Hb F content was 0.98*/ (normal 0.2–1 0*/) the Hb A_2 concentration 2.3*/ (normal 1.6–3 0*/)

The abnormal hemoglobin was isolated on starch block electrophoresis. Its separated heme-free polypeptide chains showed a normal electrophoretical migration rate of the beta chains and an increased migration of the alpha chains. The separation of the tryptic digest of the abnormal alpha chains revealed an abnormal αT-9 By means of the amino acid analysis a replacement of leucine by arginine was located in position α86 (F7). This substitution has not been described so far The new variant is called Hb Moabit with reference to the Moabit Hospital in West Berlin where the patient was hospitalized. In the patient s erythrocytes a greatly increased production of Heinz bodies after transitory methemoglobin formation was observed. The P_{50} value of the erythrocytes was found to be 30 6 mm Hg (normal 26 4–29_ mm Hg) indicating a slightly reduced oxygen affinity of the red cells.

Discussion

The amino acid F 7 is next to the proximal histidine F 8 and participates in the heme contact. A substitution of F 7 in the beta chains occurs in Hb Sabine, the variant β91 Leu→Pro described by *Schneider et al.* [9] in 1969

A replacement of a nonpolar by a polar amino acid in the interior of the heme pocket is known to weaken the heme binding probably by allowing a water molecule to enter into the normally hydrophobic heme pocket. Moreover the residue F 7 is located in a helical part of the polypeptide chain molecule where arginine as well as proline will impair the helical conformation [3 4] It is remarkable that the substitution αF 7 Leu→Arg causes only a mild, nearly compensated hemolytic anemia, while the replacement βF 7 Leu→Pro produces a severe hemolytic disorder

megaly and a nearly compensated hemolytic syndrome The hemoglobin values ranged from 12 4 to 14 0 g/dl with erythrocyte counts between 4.23 and 4 78 × 10¹²/l The MCH values varied from 28 6 to 30 1 pg There was a high reticulocytosis of about 6–10% (253 3 × 10⁹/l to 473 0 × 10⁹/l) The erythrocytes contained some typical Heinz bodies.

In smears of peripheral blood an anisocytosis poikilocytosis and hypochromia of the erythrocytes were found No spherocytes, ovalocytes or target cells could be seen. A slight thrombocytopenia probably due to the enlarged spleen was present. The leukocyte counts were normal Bone marrow smears revealed a markedly increased erythropoiesis. The iron content of the bone marrow was normal.

The osmotic fragility of the erythrocytes was found to be in the normal range with hemolysis between 0 52 and 0.36% NaCl The direct and indirect Coombs test was negative Extremely low haptoglobin concentrations of less than 0 01 g/l were found The serum values of iron, unsaturated iron binding capacity transferrin and bilirubin were normal. Pigments in the urine could not be detected, neither during the day nor at night.

⁵¹Cr labelled red cells revealed a markedly shortened life span with a half life of 14 days (normal 24–35 days). In body surface measurements of the ⁵¹Cr activity over liver heart, and spleen, the surplus counts over the spleen were greatly increased (fig. 1)

Some red cell enzymes showed an increased activity due to the reduced mean cell age i e glucose-6-phosphate dehydrogenase (26 IU/normal 12–22) 6-phosphogluconate dehydrogenase (22 IU/normal 10–16) hexokinase (2.8 IU/normal 0 8–2.0) and pyruvic kinase (114 IU/normal 24–56) The phosphohexose isomerase was normal

Hemoglobin Investigation

On starch block electrophoresis at pH 8 6 a slow moving abnormal component was noted (fig. 3) The abnormal hemoglobin migrated slightly faster than Hb S and represented about 15% of the total hemoglobin In isopropanol 12% of the hemoglobin precipitated while in the heat stability test the precipitation amounted to 21% in-

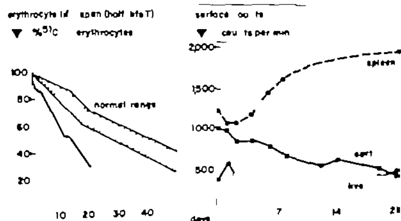


Fig. 1 Erythrokinetic study with ⁵¹Cr

Acta haemat. 61: 125–129 (1979)

Increased RNA and Heme Synthesis in Mouse Erythroid Precursors by Parathyroid Hormone

J. Levi, H. Bessler, I. Hirsch and M. Djaldetti[1]

Nephrology Unit, Department of Medicine 'B' and Hematology Clinic, Hasharon Hospital, Petah Tiqva, and Tel Aviv University Medical School, Tel Aviv

Key Words. Parathyroid hormone · RNA synthesis · Heme synthesis · Embryonic mouse liver · Erythroid precursors · Hyperparathyroidism · Anemia

Abstract. The *in vitro* effect of parathyroid hormone (PTH) on RNA and heme synthesis by embryonic mouse liver erythroid precursors was examined. PTH produced a dose-dependent effect on RNA synthesis. A maximal increase of 60 ± 16% ($p < 0.02$) was observed with 1.0 U PTH/ml, whereas with higher concentrations a significant decline was found. Furthermore, PTH stimulated heme synthesis after 24 h of incubation. The maximal enhancement of 32 ± 7% ($p < 0.01$) was observed with 0.5 U PTH/ml, a lower effect was obtained with 1.0 U PTH/ml, while 2.0 U PTH/ml caused a pronounced decrease of heme synthesis. These data indicate that PTH affects directly the erythroid precursors by a mechanism similar to that of erythropoietin. The inhibitory effect on the RNA synthesis observed with large doses of PTH may explain at least one of the causes of the anemia reported in patients with primary hyperparathyroidism.

Parathyroid hormone (PTH) and calcium were shown to stimulate lymphopoiesis and erythropoiesis [10–12]. Removal of the parathyroid gland caused a marked reduction of the mitotic activity in rat bone marrow cells, which in turn was followed by a pronounced decrease in the size of marrow nucleated cells [14]. Administration of either PTH or calcium to rats, followed by elevation of plasma calcium concentration, caused an increased reticulocyte production and radioactive iron incorporation in the red blood cells [12]. Since the ability of PTH and calcium to stimulate erythropoiesis occurred in both nephrectomized and polycythemic rats [12], it was concluded that this effect was not due to a secondary release of renal erythropoietic factor or an activation of circulating plasma erythropoietin. It was suggested that calcium and PTH may affect erythropoiesis by a direct nonspecific mitogenic action on the bone-marrow cells [13].

The aim of the present work was to ex-

[1] Established Investigator of the Chief Scientist' Bureau, Ministry of Health, Israel.

In Hb Moabit the amino acid replacement in the heme pocket is followed by an increased formation of Heinz bodies *in vivo* as well as in red cells exposed *in vitro* to oxidizing agents. Heinz bodies contain globin subunits with S–S bonds and attach to the inner surface of the red cell membrane. This process is at least partially responsible for the shortened red cell life span.

In the propositus erythrocytes P_{50} was slightly increased above the normal range. However the investigation of the cooperativity of Hb Moabit was not possible since blood samples were no longer available. The patient left Berlin to return to his family in Turkey

References

1 Betke, K. Marti H. R and Schlicht, I Estimation of small percentages of foetal haemoglobin. Nature Lond *184* 1877 (1959)

2 Beutler E. Red cell metabolism 2nd ed. (Grune & Stratton, New York 1975)

3 Bunn, H. F Forget B. G and Ranney H. M Human hemoglobins. (Saunders, Philadelphia 1977).

4 Carrell, R. W and Kay R. A simple method for the detection of unstable haemoglobins. Br J Haemat. *23* 615–619 (1972)

5 Huisman, T H. J and Jonxis, J H P The hemoglobinopathies (Dekker New York 1977).

6 Liu, C S. Simplified method for the identification of anomalous hemoglobin chains. Clinica chim. Acta *47* 315–316 (1973)

7 Marti, H R. and Scherrer R. Screening methods for haemoglobins with high oxygen affinity Abstr Int. Soc. of Haematol., Eur Afric. Div Meet., Istanbul 1977 p. 278.

8 Marti, H R., Winterhalter K. H., Di Iorio, E. E. Lorkin, P A., and Lehmann, H Hb Altdorf α_2 β_2 135 (H13) Ala→Pro: a new electrophoretically silent unstable haemoglobin variant from Switzerland. FEBS Lett. *63* 193–196 (1976)

9 Schneider R. G., Ueda S. Alperin, J B Brimhall, B and Jones, R. T., Hemoglobin Sabine beta 91 (F7) Leu→Pro. An unstable variant causing severe anemia with inclusion bodies. New Engl J Med. *280* 739–745 (1969)

Received July 13 1978
Accepted. August 18, 1978

A. Knuth,
Sloan-Kettering Institute for Cancer Research,
1275 York Avenue, New York,
NY 10021 (USA)

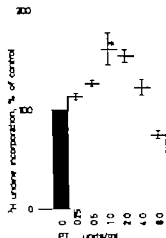


Fig. 1. Effect of PTH on RNA synthesis by 12th-day embryonic mouse liver erythroblasts. 1.0 ml of cell suspension (5 × 10⁶ cells) was incubated without and with different concentrations of the hormone for 2 h in the presence of 5 μCi ³H uridine. * = p < 0.02.

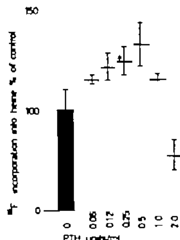


Fig. 3. Effect of PTH on heme synthesis by 13th-day embryonic mouse liver erythroblasts. 1.0 ml of cell suspension (1 × 10⁶ cells) was preincubated without and with different concentrations of the hormone for 22 h. 2 μCi ⁵⁹Fe were added and an additional incubation of 2 h was carried out. * = p < 0.01.

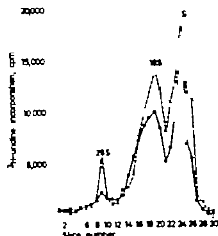


Fig. 2. Polyacrylamide gel electrophoresis of 12th-day embryonic mouse liver erythroblasts RNA after incubation without and with PTH. 1 ml of erythroblasts' suspension (1 10⁶ cells) was incubated for 2 h without and with 1.0 U PTH ml and with 10 μCi ³H-uridine. – PTH, 1.0 U/ml ● = control

22 h before addition of the radioactive iron resulted in progressive stimulation of heme synthesis (fig. 3). The maximal enhancement of 37 ± 7* (p < 0.01) was observed with 0.5 U/ml, a lower effect was obtained with 1.0 U/ml, while 2.0 U/ml caused a pronounced decrease of the erythroblasts heme synthesis. Preincubation of the cells with the same concentrations of the hormone for shorter periods (1 2, 3 or 4 h) did not have any effect on heme synthesis.

Discussion

Administration of PTH to experimental animals has been shown to produce a stimulatory effect on cell proliferation and erythropoiesis [9 10, 1., 13 19] whereas the removal of the parathyroid glands caused a

amine the *in vitro* effect of PTH on the synthetic capacity of the embryonic mouse liver erythroblasts for RNA and heme

Materials and Methods

Animals and Cells

C_5 Bl/6J mice were hormonally primed and mated according to *Southa d et al* [17] 12th and 13th-day embryonic livers were removed, placed in Eagle's minimal essential medium (MEM Gibco, Grand Island, N Y) containing 1% penicillin and streptomycin, and kept a 4 C in air containing 5% CO_2. Pooled livers were cut in small pieces, and cell suspension was obtained by repeatedly squeezing the tissue through a siliconized, fine-tipped Pasteur pipette as previously described [5], except that the liver fragments were not trypsinized

RNA Synthesis

5×10^6 12th-day embryonic liver cells suspended in 1 ml of culture medium were incubated for 2 h without and with different concentrations of PTH and 5 µCi of 5-^{3}H-uridine (spec. act. 3 1 Ci/mmol Radiochemical Centre, Amersham, England) at 37 C in a humidified atmosphere containing 5% CO_2. The reaction was stopped by addition of 10 ml cold 0 9% sodium chloride solution, and the macromolecules precipitated with 10% trichloroacetic acid (TCA) ^{3}H-uridine incorporation into macromolecules was determined as described previously [1]

Pa thyroid Hormo

Parathyroid hormone USP (Eli Lilly & Co., Indianapolis, Ind.), prepared from beef glands, was dissolved in culture medium at different concentrations.

Heme Synthesis

1×10^6 13th-day embryonic liver cells were suspended in 1 ml of culture medium supplemented with 5% fetal calf serum (Gibco) and incubated without and with PTH for 1 2, 3, 4 and 22 h under the conditions previously described. At the end of incubation, 2 µCi of carrier free $^{59}FeCl$ (spec. act. 0.2 mCi/ µg Fe; Radiochemical Centre, Amersham) preincubated with normal adult mouse plasma, were added in order to achieve transferrin binding. After 2 h, the heme was extracted with an organic solvent using the methods of *Teale* [18] 1.0 ml of the ketone layer was pipetted into 10 ml Insta-Gel (Packard Instrument, Ltd., Israel) and the radioactivity counted in a Tricarb liquid scintillation spectrometer (Packard, model 3390)

Acrylamide Gel Electrophoresis

3% (w/v) polyacrylamide gels were prepared according to *Loening* [8], except for a modification previously described [1]. RNA and heme synthesis determinations were examined in duplicates; variations between duplicates exceeding 10% were discarded. Blanks of nonspecific binding of the radioactive precursors at 0 °C were subtracted.

Results

Effect of PTH on RNA Synthesis

RNA synthesis of control erythroblasts ranged from 2×10^4 to 7×10^4 cpm/ (5×10^5 cells $\times$ 2 h) A dose-dependent effect of PTH on ^{3}H uridine incorporation into erythroid cells is shown in figure 1 1 0 U/ml of PTH produced maximal increase of 60 ± 16% (mean ± SE, $p < 0\ 02$) whereas with higher concentrations a decrease was observed 8 0 U PTH/ml caused a statistically significant inhibitory effect of 25 ± 3 % ($p < 0\ 02$) on erythroblasts RNA synthesis.

A stimulatory effect of 1 0 U PTH/ml on the synthesis of 28S and 18S ribosomal RNA and 4S transfer RNA is shown in figure 2

Effect of PTH on Heme Synthesis

The range of heme synthesis in control erythroblasts was 2,120–4 860 cpm/10^6 cells $\times$ 2 h Incubation of erythroblasts with increasing concentrations of PTH for

and Rifkind, R. A.. Erythropoietin effects on fetal mouse erythroid cells. I Cell population and hemoglobin synthesis. J Cell Biol. 51 585–595 (1971).
4 Col R. J and Paul, J The effect of erythropoietin on haem synthesis in mouse yolk sac and cultured foetal liver cells. J Embryol. xp. Morph 15 245–260 (1966).
5 Djaldetti, M., Chui, D Marks, P A., and Rifkind, R. A. Erythroid cell development in fetal mice: stabilization of the hemoglobin synthetic capacity J molec Biol. 50 345–358 (1970).
6 Djaldetti, M., Preisler H Marks, P A., and Rifkind, R. A. Erythropoietin effects on fetal mouse erythroid cells. II. Nucleic cid synthesis nd the erythropoietin sensitive cell. J biol. Chem. 247 731 735 (1972).
7 F lko, J M Guy J T Smith, R. E., and Marzaferri, E L.. Primary hyperparathyroidism and anemia. Archs intern. Med. 136 887–889 (1976).
8 Loening, U E. The fractionation of high-molecular-weight ribonucleic acid by polyacryl amide-gel electrophoresis. Biochem. J 102 251 257 (1967).
9 MacManus, J P Perris, A. D Whitfield, J F and Rixon, R. H. Control of cell division in thymic lymphocytes by parathyroid hormone, thyrocalcitonin, and cyclic adenosine 3'5'-monophosphate in Harris, The Fifth Leukocyte C lture Conference, pp. 125–142 (Academic Press, New York 1970).
10 Perris, A D The calcium homeostatic system as physiological regulator of cell proliferation in mammalian tissues in Nichols and Wasserman, Cellular mechanisms for calcium transfer and homeostasis, pp. 101 131 (Academic Press, New York 1971).
11 Perris, A D MacManus, J P Whitfield, J F and Weiss, L A.. The parathyroid glands and mitotic stimulation in rat bone-marrow after haemorrhage. Am. J Physiol. 220 773–778 (1971).
12 Perris, A. D and Whitfield, J F.: Calcium homeostasis and erythropoietic control in the rat. Can. J Physiol. Pharmacol. 49 22–35 (1971).
13 Rixon, R H Mitotic activity in the bone-marrow of rats and its relation to the level of plasma calcium. Curr Mod. Biol. 2. 68–74 (1968)
14 Rixon, R. H. and Whitfield, J F Hypoplasia of the bone marrow in rats following removal of the parathyroid glands. J Cell. Physiol 79 343–352 (1972).
15 Rogers, G M., Fisher J W and George, W J Renal cyclic AMP accumulation and adenylate cyclase stimulation by erythropoietic agents Am. J Physiol. 229 1387–1392 (1975).
16 Rogers, G. M., Fisher J W and George, W J The role f renal adenosine 3'5'-monophosphate in the control f erythropoietin production. Am. J Med. 58 31 38 (1975).
17 Southard, J L., Wolfe, H. G and Russel, E. S.. Artificial insemination of dystrophic mice with mixtures of spermatozoa Nature, Lond. 208. 1126 (1965).
18 Teale, F W J Cleavage of the haem-protein ll l, by acid methylethylketone Biochim. biophys Acta 35 543 (1959).
19 Whitfield, J F MacManus, J P Youdale, T and Franks, D J The rol of calcium and cyclic AMP in the stimulatory action f parathyroid hormone on thymic lymphocyte prolif eration. J Cell Physiol. 78. 355–368 (1971).

Received. April 28, 1978
Accepted: July 10, 1978

Prof. M. Djaldetti, Department of Medicine B' Hasharon Hospital, Petah-Tiqva (Israel)

rapid decline in bone marrow mitotic activity followed by marked reduction in 59Fe incorporation into the red cells and the production of reticulocytes [14]. Since the increased erythropoietic activity induced by PTH was not dependent upon erythropoietin *Perris and Whitfield* [12] concluded that PTH affects directly the erythroid precursors. On the other hand, *Rogers et al.* [15] have shown that PTH stimulates renal cortical cyclic AMP (cAMP) accumulation resulting in activation of renal erythropoietic factor and increased production of erythropoietin [16], thus providing a possible link between PTH and erythropoiesis. It appears that PTH may affect erythropoiesis directly or indirectly through the cAMP, or by both ways.

The present results suggest that PTH affects erythroid precursors in a way similar to that of erythropoietin [3, 4, 6]. Incubation of erythroid precursors with PTH for 2 h produced a stimulatory effect on RNA synthesis. Furthermore, PTH stimulated heme synthesis in the same system after 24 h of incubation, as measured by increased radio-iron incorporation into heme.

These findings provide additional information on the role of PTH in the regulation of erythropoiesis. The mechanism by which PTH stimulates cell proliferation was studied in a model system using thymus lymphocytes [19]. It was found that both PTH and calcium ions increase the cellular content of cAMP, promote the initiation of DNA synthesis and stimulate the proliferation of cultured rat thymocytes. Since cAMP itself stimulates cell proliferation, it could serve as mediator of the mitogenic actions of both PTH and calcium. Additional experiments showed that it was calcium which raised the cAMP level in cells treated with PTH [19]. Following these observations, it was concluded that the mitogenic action of PTH is primarily mediated by calcium [19].

It is conceivable that PTH affects the erythroid precursors *in vitro* by a similar mechanism.

The inhibition of both RNA and heme synthesis after incubation of erythroid precursors with large doses of PTH may explain the cases of anemia in patients with primary hyperparathyroidism [7].

Recently *Boxer et al.* [2] reported that 5.1% of the patients with primary hyperparathyroidism developed a normochromic, normocytic anemia which could not be related to blood loss, a deficiency state or anemia. After removal of the abnormal parathyroid glands the anemia improved or disappeared. Although the pathogenesis of the anemia remained obscure the authors suggested several possibilities, such as patchy fibrosis of the bone marrow, cysts or a suppressive effect of the PTH on erythropoiesis.

Acknowledgement

This work was partially supported by a grant from the Chief Scientist's Bureau, Ministry of Health, Israel.

References

1 Agam, G.; Bessler, H., and Djaldetti, M.: *In vitro* DNA and RNA synthesis by human platelets. Biochim. biophys. Acta *425*: 41–48 (1976).

2 Boxer, M.; Ellman, L.; Geller, R., and Wang, C. A.: Anemia in primary hyperparathyroidism. Archs intern. Med. *137*: 588–590 (1977).

3 Chui, D. H. K.; Djaldetti, M.; Marks, P. A.,

n human cells and the GE activity can be examined under different pathological, particularly leukemic conditions as suggested by other studies [32, 34 35] In a 48-hour culture the reversibility of GE action may be shown [21] Furthermore, by our method the maturation of the GE-blocked cells can be followed. Thus, the problem can be tackled as to whether the mitotic frequency reduced by GE in the granulocyte proliferation pool of the bone marrow will increase or decrease the transformation of granulocytic precursor cells to the mature cells. The following experiments were designed to solve this problem.

Materials and Methods

Bone Marrow Cultures

Human bone marrow cells were incubated by suspending them in RPMI 1640 medium (Gibco/Bio-Cult) with 2 mg/ml glucose, 10 μg/ml streptomycin and 10 μg/ml ampicillin at 37 °C [5].

For the suspension cultures, 5 mg GE was dissolved in 5 ml of cell suspension (? × 10⁶ cells/ml).

Preparation of Granulocyt Extracts

GE batch 257 or 83 3 polyvinylpyrrolidone (PVP) leukapheresis was induced in 19 rats to prepare ascites cells [20], yielding 210 ml cell suspension with 1 05 × 10⁷ cells/ml. The cells (85% granulocytes, 15% macrophages) were isolated by centrifugation (500 g 20 C) and washed with 20 ml phosphate-balanced salt solution (PBS, pH 7 3). Clumps were removed by filtration through steel grid (100 μm-mesh) and the cells were suspended in 700 ml PBS at cell density of 3 2 10⁸ cells/ml After shaking for 2 h at 37 C, the cell were removed by centrifugation t 1,500 g for 10 min The supernatant was further centrifuged at 13,000 g, 4 C for 70 min and the resulting supernatant was lyophilized

Evaluation of Proliferative Parameters

Pappenheim-stained smears were prepared after 6, 4 and 48 h from the suspension cultures. At least 2,000 cells and their mitoses were counted [3, 4] in each smear to determine the following proliferation-kinetic parameters:

(1) Mitotic indices, counted separately or erythroblasts (MI) and granulocytic precursors (wMI), gave the number of mitoses per 1,000 cells ($^0/_{00}$) capable of cell division. Statistical significance of the occurrence of mitoses of granuloblasts was tested by applying the χ^2-method of Pearson (with the correction of Yates)

(2) Myeloid erythroid ratios showed the relationship between the total number of granulopoetic and erythropoietic cells.

(3) Maturation curves represented the percentage of the granulopoietic or the erythropoietic precursors in an additive manner as function of the culture period.

(4) Caryological curves gave the percentage of the different mitotic phases of the observed mitoses of granulopoietic precursors or erythroblasts and indicated, when compared with the control, whether the added substance inhibited mitosis at specific stage or not

(5) Cell morphology was examined for the appearance f any cytotoxic effects compared with the controls, g pathological mitoses, cytoplasmic vacuoles and nuclear degeneration.

I theory granulocyte chalone should alter the following kinetic parameters of given bone marrow culture relative to the control culture in the following manner: Granuloblastic mitotic index should be temporarily reduced, erythroblastic mitotic index should remain unchanged, caryological curves of erythropoiesis and granulocytopoiesis should remain unchanged, maturation curves of erythropoiesis should remain unaffected, cell morphology should remain unchanged

As to the maturation curves of the granulopoietic series and of the myeloid erythroid ratio, no definite results can be expected since contradictory concepts have been put forward about the relationship between mitotic frequency and the transformation from one maturation stage to the other [3, 8, 11 12, 22, 23].

Results

Experiments were performed with bone marrow samples from 7 normals, from 9 pa-

Acta haemat. 61 130–137 (1979)

Evidence for a Rat Granulocyte Chalone Effect on the Proliferation of Normal Human Bone Marrow and of Myeloid Leukemias

Irene T M Boll K Sterry and H R Maurer

II Medical Department Municipal Hospital, Neukölln Berlin 47 and Pharmaceutical Institute, Freie Universität, Berlin

Key Words. Granulocyte chalone Rat ascites cells Human bone marrow *in vitro* Granulocytopoiesis Erythropoiesis Cytotoxicity Proliferation kinetics Chronic myelomonocytic leukemia Acute and chronic myeloid leukemia

Abstract Evidence is presented that rat ascites cell extracts, acting as granulocyte chalone, temporarily inhibit the cell doubling of granulocytopoietic precursors in suspension cultures of human bone marrow The extracts do not affect the proliferation of erythroblasts and do not show any cytotoxicity In addition a relative increase of granulocytic precursors capable of proliferation was found, suggesting an increase in the resting population of the granulocytopoietic proliferation pool due to extract treatment. However the extract capable of depressing the proliferation of the normal granulocytopoiesis did not affect the granulocytopoiesis of 7 chronic myeloid and 2 chronic myelomonocytic leukemias at the same dose level In contrast to these chronic leukemias, blast proliferation of 4 acute myeloid and monocytic leukemias was greatly depressed not only for 6 h but for the whole culture period of 48 h

Introduction

Chalones are defined as cell and tissue-specific, but species-unspecific inhibitors of cell proliferation which act in a reversible non-cytotoxic manner [19] Evidence for the effects of a physiological inhibitor of granulocytopoiesis (granulocyte chalone) has been demonstrated in an *in vitro* system [28 31 34] in which the proliferation kinetics of different hemopoietic cell series are followed simultaneously within the same experiment In order to show the species unspecificity the chalone is extracted from rat cells and tested on human cells, the granulocyte proliferation pool of which is affected. The advantage of an isolated *in vitro* culture over an *in vivo* system is that humoral and nervous feedback regulations are excluded [3]

In cultures with bone marrow cells from patients [3 5 6] the effect of a granulocyte extract (GE) apparently containing granulocyte chalone can be demonstrated directly

tients with chronic myelocytic or myelomonocytic leukemia and from 4 patients with acute myeloid leukemia.

Non-Malignant Bone Marrow Cultures

While the mitotic indices of the erythroblasts were not significantly changed by GE treatment (table I row 2), the mitotic index of the granulocytic precursors was significantly depressed after 6 h (table I row 1), but less so after 24 and 48 h [37 38]

The caryological curves of the erythroblastic mitoses did not show any changed pattern of the mitoses in the GE culture. It was not possible for granulocytopoietic pre cursors, to construct a corresponding curve, due to the small number of mitoses observed. The GE exerted no significant ef fects on the erythropoietic maturation as re vealed by the curve.

In normal granulocytopoiesis a small de crease of precursors capable of division was seen after 6–24 h due to GE treatment (fig. 1). After 48 h the number of cells ex

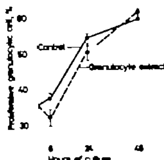


Fig. 1. Granuloblastic precursors (myeloblasts, promyelocytes, myelocytes, metamyelocytes) in percent of the total granulocytopoietic series dur ing culture time with the standard error of the mean of 9 experiments with normal bone marrow following GE treatment.

ceeded that of the control value due to lack of differentiation. The myeloid erythroid cell ratio remained unaltered. The occur rence of degeneratively changed cells having been treated by GE, as observed morphologically did not differ from that of the controls.

The relative number of monocytes, lym phocytes and plasma cells of the cultures was not altered by GE.

Leukemic Bone Marrow Cultures

The experiments with bone marrow of chronic myelomonocytic leukemias showed no GE effect on the mitotic index of the myelomoncytic series (table I, row 4), although the same GE batch was used that was active on normal bone marrow cells [37] We observed that in these leukemias more than 90% of all mitoses were confined to the promyelocyte or myelocyte stage. The mitotic indices of the erythroblasts were unchanged (table I, row 5) There was also no sign of mitotic inhibition in the caryological curves of the granulocytopoietic precursors. The differential distribution of the myelomonopoietic series showed only slight, insig nificant changes.

Likewise the mitotic indices of 7 experiments with bone marrow of chronic myeloid leukemias showed no significant difference although the values with GE were somewhat lower than those of the controls (table I, row 3) 4 bone marrow samples from Busulfan-treated chronic myeloid leukemias showed the same behavior with GE as 3 untreated cases. In the Busulfan-treated chronic myeloid leukemias, the insignificant decrease of the mitotic index was followed by a decrease of granulocytopoietic precur sors over the total observation time of 48 h [36] Erythroblasts scarcely showed mitoses

Table I. Mean mitotic indices in per mill (‰) of human marrow cell cultures

Row No	Hemopoietic cells	Hours *in vitro* 0 h	6 h C	6 h GE	24 h C	24 h GE	48 h C	48 h GE
	Normal bone marrow (experiments No 1–9)							
1	Granuloblastic mitotic index	9.9	8.8	0.9	10.9	9.6	10.8	7.8
	χ^2		27.3		0.51		2.71	
	p		0.001		ø		ø	
	Significance		+		ø		ø	
2	Erythroblastic mitotic index	19.4	13.1	11.3	5.4	5.9	2.9	4.6
	χ^2		0.67		0.08		0.61	
	p		ø		ø		ø	
	Significance		ø		ø		ø	
	Chronic myeloid leukemia (experiments No 10–16) [b]							
3	Granuloblastic mitotic index	6.1	4.9	3.5	7.0	6.9	3.8	4.5
	χ		2.68		0.04		0.57	
	p		0.19		0.95		0.3	
	Significance		ø		ø		ø	
	Myelomonocytic leukemia (experiments No 17 18)							
4	Granuloblastic mitotic index	8.6	5.5	6.8	5.6	8.1	6.[illegible]	7.2
	χ^2		0.0632		0.0455		0.0[illegible]4	
	p		0.75		0.50		0.85	
	Significance		ø		ø		ø	
5	Erythroblastic mitotic index	29.3	10.0	12.2	9.3	16.1	10.6	6.7
	χ		0.0955		0.4137		0.0228	
	p		0.80		0.83		0.83	
	Significance		ø		ø		ø	
	Acute myeloid or monocytic leukemia (experiments No 19–22)							
6	Granuloblastic mitotic index	18.1	12.9	5.4	12.0	4.6	10.6	4.6
	χ		17.89		12.66		5.70	
	p		0.0000[illegible]		0.0004		0.015	
	Significance		+		+		(+)	

C = Control GE = cultures treated with granulocyte extract of rat ascites cells p = probability
3 untreated 4 Busulfan-treated.

tients with chronic myelocytic or myelomonocytic leukemia and from 4 patients with acute myeloid leukemia.

Non-Malignant Bone Marrow Cultures

While the mitotic indices of the erythroblasts were not significantly changed by GE treatment (table I row 2), the mitotic index of the granulocytic precursors was significantly depressed after 6 h (table I, row 1), but less so after 24 and 48 h [37 38]

The caryological curves of the erythroblastic mitoses did not show any changed pattern of the mitoses in the GE culture. It was not possible, for granulocytopoietic precursors, to construct a corresponding curve, due to the small number of mitoses observed. The GE exerted no significant effects on the erythropoietic maturation as revealed by the curve.

In normal granulocytopoiesis a small decrease of precursors capable of division was seen after 6–24 h due to GE treatment (fig. 1). After 48 h the number of cells exceeded that of the control value due to lack of differentiation. The myeloid/erythroid cell ratio remained unaltered. The occurrence of degeneratively changed cells having been treated by GE, as observed morphologically did not differ from that of the controls.

The relative number of monocytes, lymphocytes and plasma cells of the cultures was not altered by GE.

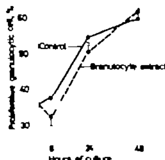


Fig. 1. Granuloblastic precursors (myeloblasts, promyelocytes, myelocytes, metamyelocytes) in percent of the total granulocytopoietic series during culture time with the standard error of the mean of 9 experiments with normal bone marrow following GE treatment.

Leukemic Bone Marrow Cultures

The experiments with bone marrow of chronic myelomonocytic leukemias showed no GE effect on the mitotic index of the myelomoncytic series (table I, row 4), although the same GE batch was used that was active on normal bone marrow cells [37] We observed that in these leukemias more than 90% of all mitoses were confined to the promyelocyte or myelocyte stage The mitotic indices of the erythroblasts were unchanged (table I, row 5) There was also no sign of mitotic inhibition in the caryological curves of the granulocytopoietic precursors. The differential distribution of the myelomonopoietic series showed only slight, insignificant changes.

Likewise, the mitotic indices of 7 experiments with bone marrow of chronic myeloid leukemias showed no significant difference, although the values with GE were somewhat lower than those of the controls (table I, row 3) 4 bone marrow samples from Busulfan-treated chronic myeloid leukemias showed the same behavior with GE as 3 untreated cases. In the Busulfan-treated chronic myeloid leukemias, the insignificant decrease of the mitotic index was followed by a decrease of granulocytopoietic precursors over the total observation time of 48 h [36] Erythroblasts scarcely showed mitoses

in the controls because of the extreme hypoplasia of erythropoiesis found in these cases.

Two experiments with bone marrow samples of acute myeloid leukemia and two of acute monocytic leukemia showed identical results (table I row 6) The addition of GE significantly decreased the mitotic indices not only after 6 h but even up to 48 h of culture The caryological curve remained unaltered suggesting a persisting decrease of cell reduplication.

Discussion

Human bone marrow cells can be cultured in a defined medium for 48 h without *significantly reducing the mitotic indices* of the granulocytopoietic and the erythropoietic series and without changing the form of the caryological curves [5] This was fully confirmed by our control experiments.

The experiments reported above were performed with crude GEs obtained by PVP leukapheresis from rat ascites cells, which have previously been shown to meet the requirements for a granulocyte chalone by the diffusion chamber technique and the agar culture system [20 24] and did not decrease the stimulation of human blood lymphocytes with phytohemagglutinin The addition of this GE significantly depressed the mitotic indices of the normal human granulocytic precursors after 6 h without significantly affecting the mitotic indices after 24 and 48 h The mitotic indices of the erythroblasts were not changed throughout the whole incubation period (table I) This finding suggests that rat GEs temporarily depress the mitotic frequency of the human granulocytic proliferation pool without being cytotoxic.

In the GE-containing cultures, a relative increase of the granuloblastic precursors was seen after 48 h (fig 1) whereas the more mature cell types and granulocytes developing by differentiation from the precursor cells in the controls decreased with incubation time Taking the primary decreased mitotic index into account (table I), this means an increase of the inactive proliferation pool [3 11 23] These results are not consistent with the interpreation by *MacKittle and McCarthy* [22] and others [8 12] who argue that differentiation into the maturation pool depends only on the time available for these processes. The increase of the cells capable of cell doubling in our experiments with non malignant bone marrow indicates that a minimal number of cell divisions is obviously needed for complete differentiation to PMNs

In the circuit between the granulocytic proliferation and reserve pools, the chalone concentration decreases the mitotic frequency and hence reduces the input into the reserve pool (fig 2) In this feedback system all factors capable of stimulating the release will interfere since they will reduce the reserve pool (leukocytosis-inducing factor leukocyte releasing factor leukopoetin G granulomovin) [*2 16 25 30*] These factors predominantly affect the PMNs, because the capacity to migrate is a prerequisite for the release [6]

Several authors have suggested that, in addition to this negative feedback regulation by chalone stimulating factors (colony stimulating factor CSF) interfere with the regulation of granulocytopoiesis [7 9 10 14 15 18 25 26 29] although there is as yet no real proof for a physiological *in vivo* effect of the *in vitro* acting CSF Several experimental data suggest a relationship be-

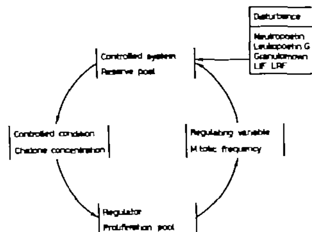


Fig. 2. Cybernetic diagram of negative feedback regulation by granulocyt chalone and interfering substances. LIF = Leukocytosis-inducing factor LRF = leukocyte-releasing factor

tween CSF and granulocyte chalone [1 27] *Haskill et al* [17] showed that human bone marrow cells are readily able to form colonies in agar culture when the apparent chalone-producing cells are removed from the culture.

It seems possible that such an antagonism did play a role in the experiments with bone marrow from myelomonocytic leukemias, since such leukemic cells are known to produce considerable quantities of CSF [13 25] thus overriding the effect of the added granulocyte chalone. Likewise, in bone marrow cultures of drug-treated and untreated chronic myelocytic leukemias the use of GE provided no significant changes. In chronic myelocytic and myelomonocytic – untreated – leukemias [4] maturation is generally decreased and further delayed by GE.

Experiments with rat GE on cells of acute myeloid leukemias are of special interest, since *Rytömaa and Kiviniemi* [32, 33] achieved regressions of a rat chloroleukemia by chalone treatment, and recently reported on preliminary clinical observations using bovine GE [34]

Our results demonstrate the same effect of depression on the leukemic granulocyte mitotic index as on normal granuloblasts, but without reversibility The reversibility was also lacking in normal bone marrow if temporarily all granulocytes were lacking in consequence to a cytotoxic depression [38] For this reason we suggest that the granulocytes inactivate the chalone or secrete so much stimulating activity as to override the inhibiting effect. The deficiency of mature granulocytes exerts a longer lasting effect of GE on the proliferation of acute myeloid and monocytic leukemia than on normal bone marrow granulocytopoetic series. Moreover these experiments support the view that GE not only affects the granulocytopoietic proliferation but also the leukemic stem cell pool.

Acknowledgements

The skillful technical assistance of Miss *C. Demeyer* and Miss *J. Heiss* are gratefully acknowledged. The work was supported by the "Deutsche Forschungsgemeinschaft

in the controls because of the extreme hypoplasia of erythropoiesis found in these cases

Two experiments with bone marrow samples of acute myeloid leukemia and two of acute monocytic leukemia showed identical results (table I row 6) The addition of GE significantly decreased the mitotic indices, not only after 6 h, but even up to 48 h of culture The caryological curve remained unaltered, suggesting a persisting decrease of cell reduplication.

Discussion

Human bone marrow cells can be cultured in a defined medium for 48 h without *significantly reducing the mitotic indices* of the granulocytopoietic and the erythropoietic series and without changing the form of the caryological curves [5] This was fully confirmed by our control experiments

The experiments reported above were performed with crude GEs, obtained by PVP leukapheresis from rat ascites cells, which have previously been shown to meet the requirements for a granulocyte chalone by the diffusion chamber technique and the agar culture system [20 24] and did not decrease the stimulation of human blood lymphocytes with phytohemagglutinin The addition of this GE significantly depressed the mitotic indices of the normal human granulocytic precursors after 6 h without significantly affecting the mitotic indices after 24 and 48 h The mitotic indices of the erythroblasts were not changed throughout the whole incubation period (table I) This finding suggests that rat GEs temporarily depress the mitotic frequency of the human granulocytic proliferation pool without being cytotoxic.

In the GE-containing cultures, a relative *increase of the granuloblastic precursors* was seen after 48 h (fig 1) whereas the more mature cell types and granulocytes developing by differentiation from the precursor cells in the controls decreased with incubation time Taking the primary decreased mitotic index into account (table I), this means an increase of the inactive proliferation pool [3 11 23] These results are not consistent with the interpreation by *MacVittie and McCarthy* [22] and others [8 12] who argue that differentiation into the maturation pool depends only on the time available for these processes. The increase of the cells capable of cell doubling in our experiments with non-malignant bone mar*row indicates that a minimal number of cell* divisions is obviously needed for complete differentiation to PMNs.

In the circuit between the granulocytic proliferation and reserve pools, the chalone concentration decreases the mitotic frequency and hence reduces the input into the reserve pool (fig 2) In this feedback system all factors capable of stimulating the release will interfere since they will reduce the reserve pool (leukocytosis-inducing factor leukocyte releasing factor leukopoetin G granulomovin) [2 *16 25 30*] These factors predominantly affect the PMNs, because the capacity to migrate is a prerequisite for the release [6]

Several authors have suggested that in addition to this negative feedback regulation by chalone stimulating factors (colony stimulating factor CSF) interfere with the regulation of granulocytopoiesis [7 9 10 14 15 18 25 26 29] although there is as yet no real proof for a physiological *in vivo* effect of the *in vitro* acting CSF Several experimental data suggest a relationship be-

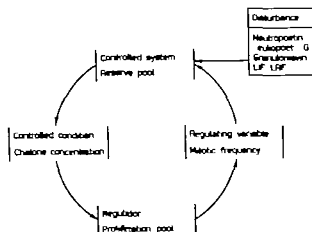


Fig. 2. Cybernetic diagram of negative feedback regulation by granulocyte chalone and interfering substances. LIF = Leukocytosis-inducing factor; LRF = leukocyte-releasing factor

tween CSF and granulocyte chalone [1 27] *Haskill et al* [17] showed that human bone marrow cells are readily able to form colonies in agar culture when the apparent chalone-producing cells are removed from the culture.

It seems possible that such an antagonism did play a role in the experiments with bone marrow from myelomonocytic leukemias, since such leukemic cells are known to produce considerable quantities of CSF [13 25] thus overriding the effect of the added granulocyte chalone. Likewise, in bone marrow cultures of drug-treated and untreated chronic myelocytic leukemias the use of GE provided no significant changes. In chronic myelocytic and myelomonocytic – untreated – leukemias [4], maturation is generally decreased and further delayed by GE.

Experiments with rat GE on cells of acute myeloid leukemias are of special interest, since *Rytömaa and Kiviniemi* [3., 33] achieved regressions of a rat chloroleukemia by chalone treatment, and recently reported on preliminary clinical observations using bovine GE [34]

Our results demonstrate the same effect of depression on the leukemic granulocyte mitotic index as on normal granuloblasts, but without reversibility The reversibility was also lacking in normal bone marrow If temporarily all granulocytes were lacking in consequence to a cytotoxic depression [38] For this reason we suggest that the granulocytes inactivate the chalone or secrete so much stimulating activity as to override the inhibiting effect. The deficiency of mature granulocytes exerts a longer-lasting effect of GE on the proliferation of acute myeloid and monocytic leukemia than on normal bone marrow granulocytopoietic series. Moreover these experiments support the view that GE not only affects the granulocytopoietic proliferation but also the leukemic stem cell pool.

Acknowledgements

The skillful technical assistance of Miss *C. Demeyer* and Miss *I. Heuer* are gratefully acknowledged. The work was supported by the 'Deutsche Forschungsgemeinschaft'

References

1 Aardal N P Laerum, O D Pankovits, W R., and Maurer H R. Inhibition of agar colony formation by partially purified granulocyte extracts (chalone) Virchows Arch. Abt. B Zellpath. 24 27–39 (1977).

2 Bierman, H R Characteristics of leukopoetin G in animals and man. Ann. N Y Acad. Sci. *113* 753 (1964).

3 Boll, I Granulocytopoese unter physiologischen und pathologischen Bedingungen (Springer Berlin 1966)

4 Boll I Leitfaden der cytologischen Knochenmarkdiagnostik Hämatologie und Bluttransfusion 2. Aufl (Lehmanns, München 1973)

5 Boll, I Collmann, H und Aust, C. Quantitative Bestimmung der Proliferationskinetik des menschlichen Knochenmarks während drei Kulturtagen. Blut *31* 201 (1975)

6 Boll, I Granulocytopoese – Morphologie, Physiologie Kinetik und Funktion in Begemann Handbuch der inneren Medizin, vol. II/3 p. 193 (Springer Berlin 1976)

7 Bradley T R. and Metcalf D The growth of mouse bone marrow cells *in vitro* Aust. J exp Biol. med. Sci *44* 287 (1966)

8 Bullough, W S. and Rytömaa, T Mitotic homeostasis. Nature, Lond. *205* 573 (1965)

9 Chervenik, P A and LeBuglio A F Human blood monocytes stimulating of granulocyte and mononuclear colony formation *in vitro* Science *178* 164 (1972)

10 Cline, M J and Golde, D W Production of colony-stimulating activity by human lymphocytes. Nature Lond. *48* 703 (1974)

11 Cronkite, E. P and Vincent, P C Granulocytopoiesis in Haematopoietic cellular proliferation, p 211 (Grune & Stratton, New York 1970)

12 Frankfurt O S Epidermal chalone. Effect on cell cycle and on development of hyperplasia. Expl Cell Res. *64* 140 (1971)

13 Golde, D W Rothman, B and Cline, M J *Production of colony-stimulating factor by* malignant leukocytes. Blood *43* 749 (1974).

14 Golde, D W and Cline M. J Identification of the colony stimulating cell in the human peripheral blood J clin. Invest. *51* 2981 (1974)

15 *Golde, D W and Cline, M J Regulation of* granulopoiesis. New Engl. J Med. *291* 1388 (1974).

16 Gordon A. S. Handler E. S. Siegel, C. D et al. Plasma factors influencing leukocyte release in rats. Ann. N Y Acad. Sci. *113* 766 (1964)

17 Haskill J S. McKnight, R D and Galbraith, P R. Cell-cell interaction *in vitro* studied by density separation of colony-forming, stimulating and inhibiting cells from human bone marrow *Blood 40 394 (1972).*

18 Iscove, N N Senn, J S. Till, J E. and McCulloch E. S. Colony formation by normal and leukemic human marrow cells in culture: effect of conditioned medium from human leukocytes. Blood *37* 1 (1971).

19 Iversen, O H History of chalones in Houck, Chalones, pp 37–69 (North Holland, Amsterdam 1976).

20 Laerum O D and Maurer H. R.. Proliferation kinetics of myelopoietic cells and macrophages in diffusion chambers after treatment with granulocyte extracts (chalone). Virchows Arch. Abt. B Zellpath. *14* 293 (1973)

21 Lord, B I Cercek, L. Shah, G P and Lajtha, L. G Inhibitors of haemopoietic cell proliferation reversibility of action. Br J Cancer *29* 407 (1974)

22 MacVittie, T J and McCarthy K. F Inhibition of granulopoiesis in diffusion chambers by a granulocyte chalone Exp Hematol., Copenh. *2* 182 (1974)

23 Maloney M A. Patt H M and Lund, J E. Granulocyte dynamics and the question of ineffective granulopoiesis. Cell Tiss. Kinet. *4* 201 (1971)

24 Maurer H R Weiss, G and Laerum, O D Starting procedures for the isolation and purification of granulocyte chalone activities. Blood *33* 161 (1976)

25 Metcalf D and Moore M A S Haemopoietic cells (North-Holland, Amsterdam 1971).

26 *Metcalf D Regul tion of granulocyte* and monocyte-macrophage proliferation by colony stimulati g factor (CSF) A review Exp. Hematol Copenh. *1* 185 (1973).

27 Muller Berat, C. N Laerum, O. D and Maurer H. R.. Chalone inhibition of the com-

mitted stem cell to granulopoiesis in vitro. 6th Meet. Eur. Study Group for Cell Prolif., Moscow 1973, p. 41.
28 Paukovits, W R.: Hemmung der DNA-Synthese unreifer Knochenmarkzellen durch spezifische, physiologische Inhibitoren; in Stacher, Leukämien und Lymphome, pp. 62 (Urban & Schwarzenberg, München 1972).
29 Robinson, W A. and Mangalik, A.. Regulation of granulopoiesis: positive feed-back. Lancet ii: 742 (1972).
30 Rohenstroth-Bauer G Gostomzyk, G. J and Feerer C. Zur Chemie des granulocytenanschwemmenden Faktors der Milz. Abstr Xth Congr Int. Soc. Haematol., Stockholm, H: 25 (1964).
31 Rytömaa, T and Kiviniemi, K.. Regulation system of blood cell production; in Teir and Rytomaa, Control of cellular growth in adult organism, pp. 106 (Academic Press, London 1967).
32 Rytömaa, T and Kiviniemi, K.. Control of DNA duplication in rat chloroleukemia by means of the granulocytic chalone. Eur J Cancer 4 595 (1968).
33 Rytömaa, T and Kiviniemi, K. Regression of generalized leukemia in rat induced by the granulocytic chalone. Eur J Cancer 6. 401 (1970).
34 Rytömaa, T Biology of the granulocyte chalone' in Houck, Chalones, pp. 289 (North-Holland, Amsterdam 1976).
35 Rytömaa, T., Vilpo, J A. Levanto, A., and Jones, W A.. Effect of granulocyte chalone on acute and chronic granulocytic leukemia in man. Report of seven cases. Scand. J Haemat., suppl. 27 (1976).
36 Schöne, E.: Die Einwirkung eines Granulocyten-Chalon-Extraktes auf menschliches Knochenmark in vitro von Patienten mit chronisch myeloischer Leukämie; Diss. (in preparation).
37 Sterry K. Die Wirkung von Granulozyten-Chalon-Extrakt auf proliferationskinetische Parameter von menschlichen Knochenmark kulturen, Diss. Berlin (1978).
38 Wollert, K. Die Einwirkung von Granulozytenchalon auf Knochenmark-Suspensionskulturen regenerierender toxischer Agranulozytosen; Diss. Berlin (1978).

Received: February 17 1978
Accepted: August 18, 1978

Prof. Dr Irene Boll, Rudower Strasse 56, D-1000 Berlin 47

References

1 Aardal N P Laerum, O D., Pankovits, W R., and Maurer H R Inhibition of agar colony formation by partially purified granulocyte extracts (chalone) Virchows Arch. Abt. B Zellpath. *4* 27–39 (1977)

2 Bierman, H. R. Characteristics of leukopoetin G in animals and man. Ann. N Y Acad. Sci. *113* 753 (1964).

3 Boll, I Granulocytopoese unter physiologischen und pathologischen Bedingungen (Springer Berlin 1966).

4 Boll, I Leitfaden der cytologischen Knochenmarkdiagnostik. *Hämatologie und Bluttransfusion*, 2. Aufl. (Lehmanns, München 1973).

5 Boll I Collmann, H. und Aust, C. Quantitative Bestimmung der Proliferationskinetik des menschlichen Knochenmarks während drei Kulturtagen. Blut *31* 201 (1975)

6 Boll, I Granulocytopoese – Morphologie, Physiologie Kinetik und Funktion in Begemann, Handbuch der inneren Medizin, vol II/3, p. 193 (Springer Berlin 1976)

7 Bradley T R. and Metcalf D The growth of mouse bone marrow cells *in vitro* Aust. J exp Biol. med Sci. *44* 287 (1966)

8 *Bullough, W S. and Rytömaa, T Mitotic* homeostasis. Nature Lond. *205* 573 (1965).

9 Chervenik, P A. and LeBuglio, A. F Human blood monocytes. stimulating of granulocyte and mononuclear colony formation *in vitro* Science *178* 164 (1972)

10 Cline M J and Golde, D W Production of colony-stimulating activity by human lymphocytes. Nature Lond. *248* 703 (1974).

11 Cronkite, E. P and Vincent, P C. Granulocytopoiesis in Haematopoietic cellular proliferation, p. 11 (Grune & Stratton, New York 1970).

12 *Frankfurt, O S. Epidermal chalone. Effect on* cell cycle and on development of hyperplasia. Expl Cell Res. *64* 140 (1971).

13 Golde D W Rothman, B., and Cline, M J Production of colony-stimulating factor by malignant leukocytes. Blood *43* 749 (1974).

14 Golde D W and Cline M J Identification of the colony-stimulating cell in the human peripheral blood. J clin Invest. *51* 2981 (1974)

15 Golde, D W and Cline, M J Regulation of granulopoiesis. New Engl. J Med. *291* 1388 (1974)

16 Gordon, A. S. Handler E. S., Siegel, C. D., et al.. Plasma factors influencing leukocyte release in rats. Ann. N Y Acad. Sci. *113* 766 (1964).

17 Haskill J S. McKnight, R. D and Galbraith, P R. Cell-cell interaction *in vitro* studied by density separation of colony-forming, stimulating and inhibiting cells from human bone marrow Blood *40* 394 (1972).

18 Iscove, N N Senn, J S., Till, J E., and McCulloch, E. S. Colony formation by normal and leukemic human marrow cells in culture· effect of conditioned medium from human leukocytes. Blood *37* 1 (1971).

19 Iversen, O H History of chalones, in Houck, Chalones, pp. 37–69 (North-Holland, Amsterdam 1976)

20 Laerum, O D and Maurer H R. Proliferation kinetics of myelopoietic cells and macrophages in diffusion chambers after treatment with granulocyte extracts (chalone). Virchows Arch Abt. B Zellpath. *14* 293 (1973).

21 Lord, B. I Cercek, L., Shah, G. P and Lajtha, L. G Inhibitors of haemopoietic cell proliferation reversibility of action. Br J. Cancer *29* 407 (1974)

22 MacVittie T J and McCarthy K F Inhibition of granulopoiesis in diffusion chambers by a granulocyte chalone. Exp Hematol., Copenh. *2* 182 (1974).

23 Maloney M. A. Patt H. M and Lund, J E. Granulocyte dynamics and the question of ineffective granulopoiesis. Cell Tiss. Kinet. *4* 201 (1971)

24 Maurer H. R. Weiss, G and Laerum, O. D Starting procedures for the isolation and purification of granulocyte chalone activities. *Blood 33 161 (1976)*

25 Metcalf D and Moore M. A S.. Haemopoietic cells (North Holland, Amsterdam 1971)

26 Metcalf D Regulation of granulocyte and monocyte-macrophage proliferation by colony-stimulating factor (CSF) A review Exp Hematol., Copenh. *1* 185 (1973).

27 Muller Berat, C N Laerum, O. D., and Maurer H. R. Chalone inhibition of the com-

tissues, and in some cases reduced their growth [19]

The following report describes the effects of hemin and protoporphyrin IX on protein-synthesizing capability of human granulocytes, lymphocytes and platelets.

Materials and Methods

Blood Cell Separations

Buffy coat was obtained from 450 ml venous blood of healthy volunteers. It consisted mainly of a mixture of granulocytes, lymphocytes and platelets and was separated into three equal portions, used for the preparation of pure cell populations. Granulocytes were separated by the method of *Skoog and Beck* [17], subsequent hemolysis of the residual red blood cells was carried out according to *Nelken et al.*, [12]. Lymphocytes were separated by the Ficoll-Hypaque procedure [18], and the platelets, as previously described [2]. Sterile conditions were maintained throughout.

Lymphocytes from 4 patients with chronic lymphatic leukemia (CLL) were obtained directly from the buffy coat of 10 ml venous blood. The differential count of the white blood cells is given in table I.

Protein Synthesis Assay

The protein-synthesizing activity of the cells was determined by 1-hour incubations of 1×10^6 granulocytes or lymphocytes, or 2-hour incubations of 5×10^7 platelets in 1 ml Eagle's minimal essential medium (MEM) without *L*-leucine and supplemented with 10 μCi ^{3}H-leucine (Radiochemical Centre, Amersham, England). ^{3}H-leucine incorporation was followed as previously described [1].

Studies of Hemin Effects

The short-term hemin effect was studied by addition of freshly dissolved hemin in the incubation mixture for protein synthesis assay in the 1-hour incubations. Hemin (Sigma Chemical Corp. St. Louis, Mo.) stock solutions were prepared by dissolving 10 mg hemin in 0.001 *M* NaOH, and diluted in phosphate-buffered saline.

Long-term hemin effects were studied in 24-hour cultures of 5×10^6 lymphocytes or granulocytes, or 5×10^8 platelets in MEM containing 15% fetal calf serum (FCS, Gibco, Grand Island, N.Y.), and streptomycin 100 μg/ml, penicillin 100 U/ml, mycostatin 100 μg/ml. The appropriate hemin solutions were added to the culture medium. Short-term and long-term incubations were carried out in humidified atmosphere containing 5% CO_2 at 37 °C.

CLL lymphocytes were cultured for 24 h with or without 3×10^{-6} *M* hemin, then assayed for protein-synthesizing activity in an assay mixture with or without freshly dissolved hemin. In these two types of experiments the assay mixture contained 10% FCS.

Protoporphyrin IX Experiments

Free acid protoporphyrin was prepared by hydrolysis of protoporphyrin dimethyl ester (Sigma) in 6 *N* HCl according to *Falk* [3]. The acid solution was evaporated using a stream of nitrogen, redissolved in 0.001 *M* NaOH, and brought to concentration of 1 mg/ml. Appropriate amounts of stock solution were added to the short- or long-term incubations.

Results

Figure 1a depicts a representative experiment of the dose-dependent enhancement of granulocyte protein synthesis following 24 h preincubation with hemin. 3.0×10^{-6} *M* of hemin caused an increase in protein synthesis up to 179 ± 35% (mean ± SE, $p < 0.05$) of the control values. Addition of freshly dissolved doses of hemin after 24 h incubation to washed cells resuspended in medium without serum caused a dose-dependent suppression of the protein synthesis.

The inhibition reached 71 ± 5% ($p < 0.05$) at a 3.0×10^{-6} *M* concentration of hemin. The inhibition curve was parallel to the curve of protein synthesis of granulocytes incubated with hemin for 1 h only

Acta haemat. 61: 138–143 (1979)

Effect of Hemin and Protoporphyrin IX on the Protein-Synthesizing Activity of Human Granulocytes, Lymphocytes and Platelets

Z. Malik, G. Agam and M. Djaldetti[1]

Department of Medicine B and Hematology Clinic, Hasharon Hospital, Petah-Tiqva, and Tel Aviv University Medical School, Tel Aviv

Key Words. Hemin · Protoporphyrin · Protein synthesis · Granulocytes · Lymphocytes · Platelets

Abstract. The hemin effect on protein synthesis of human granulocytes, lymphocytes and platelets was examined. Hemin added to culture media without serum caused a dose-dependent inhibition of protein synthesis in all three cell types. A cell-specific enhancement of protein-synthesizing capability was observed in 24-hour cultures in the presence of *hemin and serum. A marked increase of protein synthesis was found in granulocytes,* unchanged in lymphocytes and decreased in platelets. Lymphocytes from patients with chronic lymphatic leukemia (CLL) were moderately inhibited by hemin when incubated in media containing serum, the effect being more pronounced in the presence of freshly dissolved doses of hemin. Addition of protoporphyrin IX to cell cultures resulted in a marked suppression of protein synthesis by the three cell types, in all experiments. These results confirm the importance of serum proteins in preventing the inhibitory effects of free hemin and protoporphyrin IX on blood cell protein synthesis. On the other hand, they show a cell specific enhancement of the protein-synthesizing capacity mediated by hemin.

Hemin regulation of the initiation of protein synthesis was described in cell free systems of reticulocytes, ascites and brain cells [8, 13]. It was shown that hemin participates in the recycling process of the active initiation factor MP (IF-MP). The concentration of IF-MP determines the rate of globin and protein synthesis in cell-free systems [8, 13, 14]. In intact human siderotic reticulocytes and in Friend leukemic cells, hemin enhanced specifically globin synthesis [10, 15, 20]. High concentrations of hemin, up to 5.0×10^{-4} *M* were shown to increase protein synthesis in human platelets [4]. On the other hand, hemin in concentrations up to 1×10^{-4} *M* inhibited porphyrin synthesis and reduced globin synthesis in normal reticulocytes [5, 6, 20]. Furthermore, naturally occurring and synthetic porphyrins were described to concentrate into animal tumor

1 Established Investigator of the Chief Scientist's Bureau, Ministry of Health, Israel

Table I. Effect of hemin on CLL lymphocyte protein synthesis

Patient	White blood cells number/µl	Lymphocytes %	^{3}H-leucine incorporation		
			cpm/1 10^6 cells	hemin effect (% of control)	
				1 h	24 h
R. H.	91,600	93	5,550	58	74
G. U	80,000	94	8 430	71	91
G. R.	23,400	73	4 750	75	83
M. L	18,500	90	3,070	74	86

Hemin concentration 3.0 10^{-6} M.

Protein synthesis by lymphocytes cultured for 24 h with increasing concentrations of hemin remained nearly unchanged (fig. 1b). Addition of freshly dissolved hemin to the lymphocytes for 1 h resulted in a decrease of protein synthesis down to 13 ± 1% ($p < 0.01$). This effect was parallel to the decrease in the synthetic capability found in 1-hour incubations (fig. 1b) reaching 24 ± 5% ($p < 0.001$) of control values at 3.0×10^{-4} *M* hemin.

Platelet protein synthesis was suppressed by hemin both in long and short-term incubations (fig. 1c). Nevertheless, platelets cultured for 24 h with 3.0×10^{-4} *M* hemin and assayed without any hemin were suppressed by only 49 ± 3% ($p < 0.05$), whereas addition of fresh hemin to washed platelets after 24 h incubation resulted in a further decrease of protein synthesis. At 3.0×10^{-4} *M* hemin an inhibition of 99 ± 1 ($p < 0.001$) was detected.

The effect of 3.0×10^{-6} *M* hemin on CLL lymphocyte protein synthesis is shown in table I. A suppression was observed in cells preincubated with hemin. Cells cultured without hemin, but assayed in its presence, were more suppressed.

24 h preincubation of the three types of cells with protoporphyrin IX resulted in a marked suppression of protein synthesis (fig. 2). Addition of freshly dissolved doses of porphyrin in assay mixtures resulted in a total suppression of the protein-synthesizing activity of the three types of cells.

Discussion

The present study shows that increasing *concentrations of hemin result in a dose-dependent* inhibition of protein synthesis in granulocytes, lymphocytes and platelets in absence of serum. On the other hand, long-term incubations of cells cultured in media containing serum and hemin showed increased protein-synthesizing capability for granulocytes, merely unchanged in lymphocytes or decreased in platelets. It is conceivable that this finding is a result of preventing the inhibitory effect of hemin on intact cells by the binding to serum proteins. It has been shown that hemin combines unspecifically with various proteins as well as with the hemin-specific carrier – hemopexin [7 9 11 16] The reduced protein synthesis observed in the examined blood cells induced by free hemin in medium deprived of serum

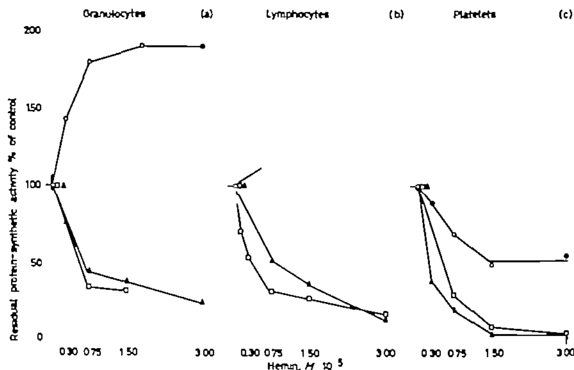


Fig. 1 Effect of hemin on granulocyte, lymphocyte and platelet protein synthesis. □ = 1-hour incubation containing cell suspensions in Eagle's MEM with ^{3}H-leucine and hemin. O = 24-hour incubation of cell suspensions in MEM with 10% FCS and hemin. Protein synthesis was assayed in absence of FCS and hemin. △ = 24-hour incubation of cell suspensions in MEM with 10% FCS and hemin. Protein synthesis was assayed in the presence of freshly dissolved hemin.

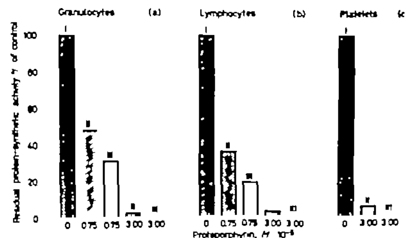


Fig. 2. Effect of protoporphyrin IX on granulocyte, lymphocyte and platelet protein synthesis. I = 1-hour assay of protein synthesis of cells in MEM with ^{3}H leucine, followed by 24 h incubation in MEM with 10% FCS. II = Preincubation of cells in MEM with 10% FCS and protoporphyrin IX. Protein synthesis assayed in absence of protoporphyrin IX. III = Preincubation of cells with protoporphyrin IX and serum. Protein synthesis assayed in MEM with freshly dissolved doses of protoporphyrin IX

cyclic 3':5' AMP independent protein kinase of the heme regulated translational inhibitor. Proc. natn. Acad. Sci. USA 73 4349–4353 (1976).
15 Ross, J and Sootner, D.. Induction of globin mRNA accumulation by hemin in cultured erythroleukemic cells. Cell 8. 513–520 (1976).
16 Sinclair P R. and Granick, S.. The transport of hemin and protoporphyrin across the plasma membrane of chick embryo liver cells in culture. Ann. clin. Res. 8. suppl. 17 pp. 250–258 (1976).
17 Skoog, W A. and Beck, W S.. Studies on fibrinogen, dextran and phytohemagglutinin methods of isolating leukocytes Blood *11* 436–454 (1956).
18 Thorsby E. and Bratlie, A.. Histocompatibility testing (Munksgaard, Copenhagen 1970).
19 Tsutsui, M. Carrano, C., and Tsutsui, E. A.. Tumor localizers: porphyrins and related compounds (unusual metalloporphyrins XXIII). Ann. N. Y. Acad. Sci. *244* 674–684 (1975).
20 White, J M and Ali, M. A. M.: Globin synthesis in sideroblastic anemia. II. The effect of pyridoxine-δ-aminolevulinic acid and haem, *in vitro*. Br J Haemat. *24* 481–489 (1973).

Received. June 13, 1978
Accepted: July 18, 1978

Prof. M. Djaldetti, Department of Medicine 'B' Hasharon Hospital, Petah-Tiqva (Israel)

emphasizes the importance of hemopexin in the prevention of the immediate inhibition of the metabolic activities by free hemin

On the other hand, incubation of blood cells with hemin in the presence of serum proteins was shown to be cell-specific. It is possible that the different responses reflect the potential effects of hemin on the initiation of protein synthesis in certain specific cells [8 13–15 20]

Protoporphyrin IX inhibited the protein synthesis of all three cell types after long term incubation Freshly dissolved doses of protoporphyrin IX in the reaction mixture increased markedly the suppression. It is possible that the lack of Fe^{+3} from the porphyrin ring altered the pattern of its effect as compared with that of hemin It may be concluded that the inhibitory effect of hemin is induced by the tetrapyrrole ring whereas the iron-containing molecule is required for the cell type specific enhancement of protein synthesis. *London et al* [8] described that hemin and not protoporphyrin enhances globin synthesis in reticulocyte cell free systems.

CLL lymphocyte protein synthesis was slightly decreased after 24 h incubation with hemin. The effect was more pronounced after addition of freshly dissolved hemin Since the incubation mixtures of CLL lymphocytes contained FCS in both short and long-term cultures, it may be assumed that the quantitative difference between the two types of experiments is due to the time course of hemin attachment to serum proteins.

References

1 Agam, G., Besler H., and Djaldetti, M *In vitro* DNA and RNA synthesis by human platelets. Biochim. biophys. Acta *425* 41–48 (1976)

2 Agam, G., Gutman, A., and Djaldetti, M. Abnormal platelet glycogen metabolism in multiple myeloma patients. Clinica chim. Acta *79*: 437–445 (1977)

3 Falk, J E., Porphyrins and metalloporphyrins (Elsevier New York 1964).

4 Freedman, M. L. and Karpatkin, S.. Requirement of iron for platelet protein synthesis. Biochem. biophys. Res. Commun. *54* 475–481 (1973).

5 Giradi, A. S. and Levere, R. D Enzymatic formation and cellular regulation of heme synthesis. Semin. Hemat. *14* 145–168 (1977).

6 Karibian, D and London, I. M.. Control of heme synthesis by feedback inhibition. Biochem. biophys. Res. Commun. *18*. 243-250 (1965)

7 Koskelo, P and Muller Eberhard, U Interaction of porphyrins with proteins. Semin. Hemat. *14* 221–226 (1977).

8 London, I. M. Clemens, M. J., Ranu, R. S Levin, D H. Cherbas, L. F and Ernst, V The role of hemin in the regulation of protein synthesis in erythroid cells. Fed. Proc. *35* 2218–2222 (1976).

9 Malik, Z. Biosynthesis of porphyrins and hemoglobin in Friend erythroleukemic cells; PhD thesis, Weizmann Institute of Science Rehovot, Israel (1977)

10 Malik, Z. Halbrecht, I., and Djaldetti, M Regulation of hemoglobin synthesis, iron metabolism and maturation of Friend leukemic cells by 5-amino levulinic acid and hemin. Differentiation (in press).

11 Morgan, W T Porphyrin-binding proteins in serum. Ann. N Y Acad. Sci. *244* 624–650 (1975).

12 Nelken, D., Gilboa-Garber N., and Gurevitch, J A method for the simultaneous separation of human thrombocytes and leucocytes. J clin. Path. *13*. 266 (1960)

13 Raffel, C., Stein, S., and Kaempfer R. Role for heme in mammalian protein synthesis. activation of an initiation factor Proc. natn. Acad. Sci. USA *71* 4020–4024 (1974)

14 Ranu, R. S. and London, I M.. Regulation of protein synthesis in rabbit reticulocyte lysates: purification and initial characterization of the

Similar results in uraemic patients undergoing RDT were reported by others [2]

The reduction of tetrazolium salts was found to be normal or increased in granulocytes of RDT patients [10, 11 29] The literature concerning the enzymatic apparatus of granulocytes in uraemia is scarce. A decreased activity of acid phosphatase accompanied by cell destruction was found in cellular elements of skin exudates [17]

In this paper an attempt is made to evaluate the activity of certain enzymes that to our knowledge have not been investigated so far in uraemia. The spontaneous and stimulated NBT reduction in granulocytes of RDT and nondialysed uraemic patients is also investigated. Such an approach can give information about the state of leukocytes in uraemic patients.

Materials and Methods

The investigations were carried out on 14 RDT (6 females and 8 males) as well as on 10 nondialysed uraemic patients treated at the Division of Nephrology 17 suffered from glomerulonephritis, 4 from nephrosclerosis, 1 from lupus nephritis, 1 from polycystic kidney disease and 1 from chronic pyelonephritis. The mean age of patients was 33.7 (16–48 years). Each RDT patient was dialysed for 6–9 h, 2–3 times weekly over the period of 1–84 months (mean 19). 'Nycotron' dialysers were used in 7 cases, 'Bellco' in 5 cases, and 'Meltec' multipoint dialysers in 2 cases, respectively In each type Cuprophan PT 150 membrane was employed.

The mean value of urea was 31.2 mmol/l (10 1 73) in nondialysed and 11 6 mmol/l (4 6–14) in dialysed patients. The level of creatinine was 960 1 μmol/l (170–.,160) and 573 μmol/l (312–890) respectively The leukocytosis in nondialysed patients was $\bar{X}$ = 8.054.5 (5,900–16,500) and $\bar{X}$ = 7,578.5 (3,300–11,500) in RDT patients. None of the patients under study was given steroids or cytostatics.

The blood from 30 healthy subjects was used as control for NBT test and blood smears from 20 persons served as control for the enzymatic reactions.

In all subjects the following reactions were performed:

(1) Test of spontaneous and latex-stimulated reduction of NBT according to *Park et al.* [24]. Granulocytes with large deeply-blue formazan deposits or cells containing highly visible blue grains were qualified as NBT-positive [25]. The test of spontaneous NBT reduction was performed once in controls and in nondialysed patients and several times in each of the RDT patients. The test of stimulated NBT reduction (Bacto-Latex 0.81 μm, Difco Labs.) was performed once in controls and nondialysed patients, and twice (immediately before and after dialysis) in RDT patients.

(2) Reaction for alkaline phosphatase was performed according to *Kaplow* [18]. The intensity of reaction was estimated semi-quantitatively using a 5-degree score method (0–4 scale, depending on the staining intensity).

(3) Acid phosphatase activity in granulocytes and lymphocytes was demonstrated according t *Barka and Anderson* [4]. The intensity of reaction in granulocytes was estimated using 4-degree score method (0–3 scale), while in lymphocytes the proportion of positive cells was established and the intensity of reactions was expressed in 3-degree scale with respect to the form of reaction product (granular or diffuse), according to the following criteria. 0 = no reaction 1 = diffuse reaction or granular reaction with 1 3 positive granules per cell, 2 = granular reaction with more than 3 positive granules per cell or a mixed, diffuse/granular reaction.

(4) Peroxidase reaction was performed according to *Graham and Karnovsky* [13]. Intensity of reaction was evaluated by 4-degree score method (0–3 scale) In 6 patients all enzymatic reactions were performed twice, in the remaining cases once, thus in dialysed patients 20 enzymatic reactions were performed altogether The blood for these reactions was collected immediately before dialysis. In nondialysed patients and healthy subjects reactions were performed only once. The activity of each enzyme was estimated in all smears by one person and evaluation was blind. Total score was obtained by multiplication of cell

Acta haemat. 61 144–149 (1979)

The Enzymatic Activities and NBT Reduction Test of Granulocytes in Untreated and Dialysed Uraemic Patients[1]

W Sułowicz, Z Komorowska Z Hanicki T Cichocki and O Smoleński

Kidney Clinic of the Institute of Internal Medicine (Head. Prof. Z. *Hanicki*) and Department of Histology (Head. Assoc. Prof. *T Cichocki*) Medical Academy Cracow

Key Words. Acid phosphatase Alkaline phosphatase Peroxidase NBT reduction test Granulocytes Uraemia Dialysis

Abstract. It was found that neutrophils in untreated uraemic patients as well as in subjects on regular dialysis treatment displayed higher activity of acid phosphatase, alkaline phosphatase and peroxidase. Spontaneous reduction of nitro blue tetrazolium (NBT) by granulocytes was also higher in both groups in comparison to controls. Stimulation with latex particles gave similar results of NBT reduction in investigated patients and controls. Lymphocytes also showed an increase in acid phosphatase activity if compared to healthy persons. It seems possible that granulocytes which take part in unspecific defense mechanisms are more active in uraemic patients due perhaps to subclinical infections.

Bacterial infections are believed to be the main cause of death in uraemia in about 20% of patients [21 22] The mechanism responsible for increased susceptibility to infections is still poorly understood. Among others, a defect in cellular immunity is particularly stressed. cutaneous reactivity is suppressed [19 26 28] homograft rejection delayed [12] and PHA induced lymphocyte blastic transformation depressed [6 16 23]

Neutrophils are the first unspecific line of defense against microbial infections. Observations on the physiological state of these cells in uraemia are still controversial. According to *Buscarini* [9] the granulocyte reserve in bone marrow in patients on repeated dialysis treatment (RDT) is decreased, while the mitotic index is slightly above normal. The elements of myeloid cell series show vacuolisation of cytoplasm and chromatolysis. The phagocytic properties of granulocytes in uraemia were reported as decreased [8 22] or normal [2, 11 14] The bactericidal function was not altered both in dialysed and nondialysed patients [14]

[1] Supported by Polish-American Coordination Committee for Scientific Cooperation, Project Officer *Benjamin T Burton*, NIH, Bethesda, MD.

Similar results in uraemic patients undergoing RDT were reported by others [2]

The reduction of tetrazolium salts was found to be normal or increased in granulocytes of RDT patients [10 11 29] The literature concerning the enzymatic apparatus of granulocytes in uraemia is scarce. A decreased activity of acid phosphatase accompanied by cell destruction was found in cellular elements of skin exudates [17]

In this paper an attempt is made to evaluate the activity of certain enzymes that to our knowledge have not been investigated so far in uraemia. The spontaneous and stimulated NBT reduction in granulocytes of RDT and nondialysed uraemic patients is also investigated. Such an approach can give information about the state of leukocytes in uraemic patients.

Materials and Methods

The investigations were carried out on 14 *RDT* (6 females and 8 males) as well as on *10* nondialysed uraemic patients treated at the Division of Nephrology 17 suffered from glomerulonephritis, 4 from nephrosclerosis, 1 from lupus nephritis, 1 from polycystic kidney disease and 1 from chronic pyelonephritis. The mean age of patients was 33.7 (16–48 years). Each RDT patient was dialysed for 6–9 h, 2–3 times weekly over the period of 1–84 months (mean 19). 'Nycotron' dialysers were used in 7 cases, 'Belleo' in 5 cases, and 'Meltec' multipoint dialysers in 2 cases, respectively. In each type a Cuprophan PT 150 membrane was employed.

The mean value of urea was 31.2 mmol/l (10.1 73) in nondialysed and 11.6 mmol/l (4.6–14) in dialysed patients. The level of creatinine was 960.1 μmol/l (170–2,160) and 573 μmol/l (312–890), respectively. The leukocytosis in nondialysed patients was $\bar{x}$ = 8,054.5 (5,500–16,500) and $\bar{x}$ = 7,578.5 (3,300–11,500) in RDT patients. None of the patients under study was given steroids or cytostatics.

The blood from 30 healthy subjects was used as a control for NBT test and blood smears from 20 persons served as control for the enzymatic reactions.

In all subjects the following reactions were performed.

(1) Test of spontaneous and latex-stimulated reduction of NBT according to *Park et al.* [24]. Granulocytes with large- deeply-blue formazan deposits or cells containing highly visibl blue grains were qualified as NBT-positive [25]. The test of spontaneous NBT reduction was performed once in controls and in nondialysed patients and several times in each of the RDT patients. The test of stimulated NBT reduction (Bacto-Latex 0.81 μm, Difco Labs.) was performed once in controls and nondialysed patients, and twice (immediately before and after dialysis) in RDT patients.

(2) Reaction for alkaline phosphatase was performed according to *Kaplow* [18]. The intensity of reaction was estimated semi-quantitatively using a 5-degree score method (0–4 scale, depending on the staining intensity).

(3) Acid phosphatase activity in granulocytes and lymphocytes was demonstrated according to *Barka and Anderson* [4]. The intensity of reaction in granulocytes was estimated using 4-degree score method (0–3 scale), while in lymphocytes the proportion of positive cells was established and the intensity of reactions was expressed in a 3-degree scale with respect to th form of reaction product (granular or diffuse), according to the following criteria. 0 = no reaction 1 = diffuse reaction or granular reaction with 1 3 positive granules per cell; 2 = granular reaction with more than 3 positive granules per cell or mixed, diffuse/granular reaction.

(4) Peroxidase reaction was performed according to *Graham and Karnovsky* [13]. Intensity of reaction was evaluated by 4-degree score method (0–3 scale). In 6 patients all enzymatic reactions were performed twice, in the remaining cases once, thus in dialysed patients 20 enzymatic reactions were performed altogether The blood for these reactions was collected immediately before dialysis. In nondialysed patients and healthy subjects reactions were performed only once. The activity of each enzyme was estimated in all smears by one person and evaluation was blind. Total score was obtained by multiplication of cell

number by their respective scale values, 100 cells of each type (neutrophils and lymphocytes) were estimated in each smear

Results

The data obtained are given in tables I and II The results of NBT reduction ex pressed by percent of granulocytes with formazan deposits were as follows The mean values of spontaneous NBT reduction in RDT patients before dialysis ($\bar{X}$ = 16.7%) and immediately thereafter ($\bar{X}$ = 17.9%) were similarly higher than in controls ($\bar{X}$ = 9.5%) In the group of nondialysed patients mean values of spontaneous NBT reduction ($\bar{X}$ = 21 6%) were also elevated and even exceeded values found in dialysed patients.

The mean values of stimulated NBT re duction in the group of dialysed patients both before ($\bar{X}$ = 45.9%) and after dialysis ($\bar{X}$ = 44 8%) were almost identical and slightly below control values ($\bar{X}$ = 50 6%) while in nondialysed patients were higher ($\bar{X}$ = 57 1%) There was an increase in peroxidase activity in the untreated uraemic patients ($\bar{X}$ = 289 60) and in the RDT patients ($\bar{X}$ = 244.35) as compared to the controls ($\bar{X}$ = 171 80)

The activity of alkaline phosphatase was higher both in RDT ($\bar{X}$ = 105.25) and in nondialysed uraemic patients ($\bar{X}$ = 112.00) in comparison to control values ($\bar{X}$ = 76 40).

Table I. Spontaneous and latex-stimulated NBT reduction in uraemic patients

NBT reduction	Controls (n = 30)	Un-treated uraemic patients	RDT before D (n = 41)	RDT after D (n = 33)
Spontaneous NBT reduction %	mean 9.5	21 6	17.9	16.7
	± SD 7 1	19 7	16.5	14.3
	range 1–30	7–60	1–60	1–68
Latex-stimulated NBT reduction %	mean 50 6	57 1	45.9	44 8
	± SD 17 8	13 7	15 6	18.8
	range 16–89	26–76	16–65	18–87

Table II. Activity of acid phosphatase, alkaline phosphatase and peroxidase in uraemic patients

Enzymes		Controls	Untreated uraemic patients	RDT
Acid phosphatase of neutrophils	mean	81 15	130.50	178.50
	± SD	62.25	51.58	38.47
	range	4–220	82 208	68–229
Acid phosphatase of lymphocytes	mean	61.05	122.50	136.15
	± SD	33.52	25 47	38.90
	range	5–124	68–168	16–170
Alkaline phosphatase of neutrophils	mean	76.40	112.00	105.25
	± SD	42.92	74.24	56.14
	range	15–142	35–237	2 212
Peroxidase of neutrophils	mean	171.80	289 67	244.35
	± SD	24.96	52.66	27 74
	range	132–225	203–317	178–290

The activity of acid phosphatase in granulocytes of both RDT ($\bar{X}$ = 178.50) and nondialysed patients ($\bar{X}$ = 130.50) was higher than in control group ($\bar{X}$ = 81 15) Similarly there was increase in phosphatase activity in lymphocytes: mean values for RDT patients were $\bar{X}$ = 136.15 and for nondialysed patients $\bar{X}$ = 122.50 while in controls $\bar{X}$ = 61 05

The percent of lymphocytes with a positive reaction for acid phosphatase was also higher in untreated uraemic patients ($\bar{X}$ = 78.1%) and RDT patients ($\bar{X}$ = 73 6%) against control values ($\bar{X}$ = 48.5 %)

Discussion

The results of several authors confirm our observations that neutrophils of RDT and nondialysed uraemic patients are functionally stimulated (2, 7 11 14 22). The evidence of other authors indicate, however that the function of granulocytes of uraemic patients is normal or even lowered [2, 5 27] It is possible that these discrepancies result from the fact that granulocytes function is influenced by several factors having antagonistic properties, e.g uraemic toxins, subclinical infections, drugs. The intracellular NBT reduction is an index of the activation of metabolic processes in neutrophils as a consequence of phagocytosis [3] *Wolman et al* [30] stated that uraemic patients without infections show normal NBT reduction. However other authors observed a higher NBT reduction in uraemic patients [10 29]. In 3 out of 14 RDT patients permanently higher spontaneous NBT reduction was noted. Only 1 patient displayed symptoms of repeated infections of respiratory origin. In 9 patients a periodic increase of NBT reduction was found without any clinical signs of infections. In 2 RDT patients low values of NBT reduction were observed.

An increase of peroxidase activity indicates a higher ability of neutrophils for production of H_2O_2 which seems to be an important factor in the killing of bacteria by halogenation [20] It can be postulated therefore, that as in the case of NBT reduction, this increase might be compensatory as the specific immunity is decreased in uraemic patients [12, 19 26, 28] It is also possible that changes in body fluid composition (higher level of solutes in small and middle molecular weight range) may directly stimulate granulocytes [15] The functional stimulation of granulocytes is also expressed by an increase of alkaline and acid phosphatase activity Our findings are consistent with observations of *Abbrederis et al.* [1] who demonstrated that alkaline phosphatase activity in granulocytes of uraemic patients is elevated.

In the light of our results it seems justifiable to conclude that in the course of uraemia the activity of granulocytes increases. It is, however difficult to determine if these changes are primary or secondary In the latter case they would result from susceptibility to infections caused by defects in specific immunological responses.

References

1 Abbrederis, K., Schmalzl, F und Dittrich, P Das Verhalten der alkalischen Leukozytenphosphatase bei Hämodialysepatienten. Med. Klin. 72. 212–215 (1977).

2 Abrutyn, E., Solomons, N. W.; Clair R. K., MacGregor R. R., and Root, R. K. Granulocyte function in patients with chronic renal failure: surface adherence, phagocytosis and

bactericidal activity *in vitro* J Infect. Dis. *135* 1–8 (1977).

3 Baechner R. L. and Nathan, D G Leukocyte oxidase: defective activity in chronic granulomatous disease. Science N Y *155* 835–836 (1967).

4 Barka, T and Anderson, P.. Histochemical methods for acid phosphatase using hexazonium pararosanilin as coupler J Histochem. Cytochem. *10* 741–753 (1962).

5 Baum, J Cestero, R. V M., and Freeman, R. B.. Chemotaxis of the polymorphonuclear leukocyte and delayed hypersensitivity in uremia. Kidney int. *7* suppl. 2, pp. 147–153 (1975).

6 Boulton Jones, J M., Vick, R. Cameron, J S., and Black, P J.. Immune responses in uremia. Clin. Nephrol. *1* 351–360 (1973).

7 Brogan, T D.. Phagocytosis by polymorphonuclear leukocytes from patients with renal failure. Br med. J *III*. 596–599 (1967).

8 Burleson, R. L.. Reversible inhibition of phagocytosis in anephric uremic patients. Surg. Forum *24* 75–77 (1973).

9 Buscarini, L. La réserve de granulocytes médullaires chez des patients soumis à l'hémodialyse périodique. Acta haemat. *56* 7–9 (1976).

10 Camppa, M. E. Rabinovich, S., and Smith, I. M. The nitroblue tetrazolium test. An evaluation of the false-positive and false-negative results. Archs intern. Med. *133* 432–436 (1974)

11 Chretin, J H. and Garagusi, V F Phagocytosis and nitroblue tetrazolium reduction in uremia. Experientia *29* 612–613 (1973).

12 Dammin, G J., Couch, N P., and Murray J E. Prolonged survival of skin homografts in uremic patients. Ann. N Y Acad. Sci. *64* 967–976 (1957).

13 Graham, R. C. and Karnovsky M. J The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J Histochem. Cytochem. *14* 291–302 (1966).

14 Gucklan, J C., Karrh, L. R. Copeland, J L., and McCoy J Phagocytosis by polymorphonuclear leucocytes in patients with renal failure on chronic hemodialysis. Tex. Rep Biol. Med. 29 193–198 (1971).

15 Hanicki, Z. Sarnecka-Keller M., Klein, A., and Slizowska, K.. Middle-sized ninhydrin positive molecules in uremic patients treated by repeated hemodialysis. Preliminary characteristics. Clinica chim. Acta *54* 47–54 (1974).

16 Hanicki, Z. Cichocki, T Sarnecka Keller M., Klein, A., and Komorowska, Z.. Influence of middle sized molecule aggregates from dialysate of uremic patients on lymphocyte transformation *in vitro* Nephron *17* 73–80 (1976).

17 Hanicki, Z., Cichocki, T., Komorowska, Z., and Smoleński, O.. Skin window test in untreated and on regular dialysis treatment (RDT) patients. Donau Symposium für Nephrologie, Linz/Donau, Österreich, pp. 79–82 (Bindernagel Friedberg/Hessen 1976)

18 Kaplow L. S.. A histochemical procedure for localising and evaluating leukocyte alkaline phosphatase activity in smears of blood and marrow Blood *10* 1023–1029 (1955)

19 Kirkpatrick, C. H., Wilson, W E., and Talmage, D W Immunologic studies in human organ transplantation. I. Observation and characterization of suppressed cutaneous reactivity in uremia. J exp Med. *119* 727–742 (1964).

20 Klebanoff, F J.. Antimicrobial mechanisms in neutrophilic polymorphonuclear leucocytes. Semin. Hemat. *12* 117–14. (1975).

21 Lowrie, E. G Lazarus, J M. Mocelin, A. J Bailey G L., Hampers, C. L., Wilson, R. E., and Merrill, J P.. Survival of patients undergoing chronic hemodialysis and renal transplantation. New Engl. J Med. *288* 863–867 (1973).

22 Montgomerie, J Z., Kalmanson, G. M., and Guze, L. B.. Renal failure and infection. Medicine, Balt. *47* 1–32 (1968)

23 Newbery W M and Sanford, J P Defective cellular immunity in renal failure: depression of reactivity of lymphocytes to phytohemagglutinin by renal failure serum. J clin. Invest. *50* 1.62–1271 (1971).

24 Park, B. H., Fikring, S. M., and Southwick, E. M.. Infection and nitroblue reduction by neutrophils: a diagnostic aid. Lancet *II* 532–534 (1968).

25 Szczepaniec, M. and Sulowicz, W.. The nitroblue tetriazolium (NBT) test in selected respiratory diseases: NBT test in the differential diagnosis of pneumonia and pulmonary throm-

boembolism. Pol. Arch. Med. Wewn. 56: 335–344 (1976).
26 Touraine, J. L., Revillard, J. P., Brochier, J. Manel, A. M., Fries, D. et Traeger, H. G. Le déficit de l'immunité cellulaire secondaire l'insuffisance rénale. Lyon méd. 224: 185–200 (1970).
27 Warden, G. D. and Maxwell, J. G. Leukocyte chemotaxis *in vitro* in chronic dialysis patients and after renal transplantation. Surg. Forum 27: 323–325 (1976).
28 Wilson, W. E., Kirkpatrick, C. H., and Talmage, D. W. Suppression of immunologic responsiveness in uremia. Ann. intern. Med. 62: 1–14 (1965).
29 Winchester, J. F., Gordon, A. M., Rowan, R. M., Lindsey, R. M., and Black, D. A.: Interpretation of the nitroblue-tetrazolium test in regularly dialysed patients. Lancet ii: 292–293 (1973).
30 Wolman, M. R., David, D. S., Brennan, B. L., Lewy, J. E., Stenzel, K. H., Rubin, A. L., and Miller, D. R.: The nitroblue-tetrazolium test. Usefulness in detecting bacterial infections in uremic and immunosuppressed renal transplant patients. Lancet ii: 289–292 (1972).

Received: January 17, 1978
Accepted: August 18, 1978

Z. Hanicki, MD, Copernicus Street 15, 31 501 Cracow (Poland)

Acta haemat. *61* 150–154 (1979)

Differential Blood Counts from Cell Concentrates

A Comparison with Routine Differential Blood Counts[1]

H. Pflieger, W. Gaus and M. Dietrich

Departments of Internal Medicine and Documentation and Statistics, University of Ulm, Ulm, and Department of Medicine, Bernhard-Nocht Institute for Naval and Tropical Diseases, Hamburg

Key Words. Differential blood counts · Routine films · Cell concentrate films

Abstract. Two methods have been compared through performing differential blood counts: the routine blood count (routine count = RC) and blood counts from films after concentrating white blood cells (cell concentrate = CC). Normal persons were examined and patients with leukocytes less than 1 000/μl of peripheral blood. The correlation coefficient between RC and CC is higher for more frequent cell types and lower for less frequent cell types. It is concluded that the preparation of CC is simple and reproducible, and that the time-saving CC method for performing differential blood counts from patients with severe leukopenia may be a worthwhile addition to routine laboratory methods.

Introduction

Several blood films have to be examined *in severely leukopenic patients in order to* obtain an accurate differential blood count. In this study two methods of performing differential blood counts are compared: (a) the routine blood count obtained from EDTA anticoagulated peripheral blood smears, (b) blood counts from films obtained after concentrating white blood cells from EDTA anticoagulated peripheral blood. The use of cell concentrates may lead to shortening of the time required for these counts.

[1] Supported by 'Deutsche Forschungsgemeinschaft' SFB 112.

Material and Methods

14 normal healthy persons (blood donors in the Blood Bank of Ulm) and 10 patients with acute leukemia were included in this study. The 10 patients with acute leukemia were examined on 26 separate occasions when they had less than 1,000 leukocytes/μl of peripheral blood.

For each examination 8 ml of venous blood were collected into EDTA (EDTA test tubes, Sarstedt). Visual white cell counts were performed using standard methods [2]. 5 blood films were prepared for routine differential counts from each specimen. Leukocyte concentrates [1] were prepared as follows: 4 ml of EDTA blood were mixed with 1 ml of neo-Plasmagel (Braun Melsungen AG) in a siliconized test tube (the internal diameter was 10 mm). This was then sealed with Parafilm (American Can Company) inverted thrice and left in a vertical plane undisturbed for

30 min at room temperature. After the sedimentation of erythrocytes was complete the supernatant then consisted of plasma, leukocytes, platelets and neo-Plasmagel. This was pipetted into a fresh silliconized test tube and centrifuged for 5 min at 400 *g*. The sediment obtained after centrifugation was resuspended in 0.5 ml of the supernatant. From this suspension films were prepared. All films – the conventional ones and the films from the leukocyte concentrates – were fixed for 10 min in absolute methanol and stained with May-Grünwald-Giemsa solution. Differential blood counts were made from the last third of the films under 400 magnification (Standard Mikroskop Carl Zeiss AG) by two laboratory technicians who each made a differential count of 500 cells.

Statistical Analysis

The correlation coefficient between the cell counts of films from CC and from RC were determined for each type of cell. For this purpose the mean was calculated from the counts made by the two laboratory technicians. In addition, the confidence limits of the correlation coefficient were calculated [3, 5].

The correlation coefficient between the counts of the two technicians in the same laboratory and its confidence limits were determined in order to give better interpretation of the correlation coefficient between CC and RC counts. The correlation coefficient between the two technicians was determined separately for the CC films and the RC films.

For each type of cell, linear regression was computed with the RC value as the 'true score variable' X and the CC values as dependent variable Y. In all these regressions the absolute term is expected to be zero, so the computation of the regression coefficient simplifies to regression coefficient = mean of Y divided by mean of X. This was performed as one cannot expect all the cell types to be equally concentrated by the leukocyte concentration procedure. As the concentration of the different cell types is not entirely uniform the correction factor is used to offset this deficiency. The corrected CC values correspond with the usual RC values.

Results

The preparation of cell concentrates is simple and reproducible. Usually there are 8–12 times as many cells in CC films than

Table I. Mean of differential blood counts from cell concentrate films (CC) and routine blood films (RC)

Type of cell	Samples from 124 blood donors			26 samples from patients with leukopenia (< 1,000 leukocytes/µl)		
	CC,	RC,	ratio CC/RC	CC,	RC,	ratio CC/RC
Segmented forms	6[illegible].91	54.96	1.145	8.96	6.66	1.347
Band forms	1.44	1.04	1.394	0.49	0.65	0.748
Metamyelocytes	0.17	0.12	1.479	0.07	0.08	0.873
Myelocytes	0.28	0.39	0.725	0.11	0.30	0.370
Promyelocytes				0.03	0.15	0.227
Lymphocytes	[illegible]6.37	32.97	0.800	87.47	87.87	0.995
Monocytes	5.10	7.26	0.702	0.27	0.82	0.335
Eosinophils	[illegible].85	[illegible].59	1.103	0.04	0.02	[illegible]
Basophils	0.86	0.68	1.265	0.14	0.1	1.181
Red precursors				1.5[illegible]	1.34	1.139
Leukemic cells				0.89	[illegible].00	0.444

Table II. Correlation coefficients (r) between RC and CC blood counts of 124 blood donors and between the counts of two laboratory technicians

Type of cells	Correlation coefficient between CC and RC			Correlation coefficient between the counts of two technicians of the same laboratory					
				CC			RC		
	r	confidence interval of r (p = 0.95)		r	confidence interval of r (p = 0.95)		r	confidence interval of r (p = 0.95)	
Segmented forms	0 70	0.59	0.78	0.77	0 69	0.83	0.65	0.53	0.74
Band forms	0.71	0.61	0 79	0.59	0.46	0.69	0.40	0.24	0.54
Lymphocytes	0.65	0.53	0 74	0 83	0.77	0.88	0.67	0.56	0.76
Monocytes	0.26	0.09	0 42	0 48	0.33	0 60	0.21	0.03	0.37
Eosinophils	0.71	0.61	0.79	0.66	0.55	0.75	0 42	0.26	0.55
Basophils	0.37	0.21	0.51	0.56	0 43	0.67	0.02	-0.16	0.19

No correlation was computed for myelocytes and metamyelocytes due to a poor distribution caused by too many zero values.

in RC films. However the different cell types do not equally concentrate. Table I shows that for example segmented forms concentrate more than monocytes. Table II shows the correlation coefficients between the RC values and the CC values and their confidence intervals for the films of the blood donors. Due to the absence of the myelocytes and metamyelocytes in many cases no satisfactory frequency distribution was found Therefore no correlation coefficients were computed for these two cell types. The correlation for the more frequent cell types (segmented forms and lymphocytes) is in the order of 0 7 Table II also indicates the correlation coefficients of the performance of the two laboratory technicians. The correlation between RC and CC is in the same order as the correlation between the two laboratory technicians. The correlation between the technicians is higher for CC than for RC for all cell types.

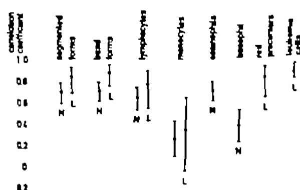


Fig. 1 Correlations between differential blood counts from RC and CC. N = blood donors L = leukopenic patients; vertical bars indicate correlation coefficient and confidence interval p = 0.95 of the correlation coefficient.

The same information is obtained from blood films made from patients with acute leukemia and severe leukopenia as shown in table III The frequency distribution of promyelocytes, myelocytes and metamyelocytes was insufficient for the computation of

Table III. Correlation coefficient ( ) in the differential blood counts from 10 patients with leukopenia (< 1,000 leukocytes/µl) obtained on 26 separate occasions

Type of cells	Correlation coefficient between CC and RC			Correlation coefficient between the counts of two technicians of the same laboratory					
				CC			RC		
		confidence interval of (p = 0.95)			confidence interval of (p = 0.95)			confidence interval of (p = 0.95)	
Segmented forms	0.85	0.69	0.93	0.86	0.71	0.94	0.97	0.93	0.99
Band forms	0.88	0.75	0.95	0.56	0.25	0.78	0.62	0.31	0.81
Lymphocytes	0.77	0.54	0.89	0.89	0.77	0.94	0.92	0.83	0.96
Monocytes	0.34	–0.05	0.64	0.34	–0.05	0.64	0.40	0.01	0.68
Red precursors	0.82	0.63	0.92	0.95	0.89	0.98	0.65	0.35	0.83
Leukemic cells	0.85	0.69	0.93	0.33	–0.07	0.64	0.86	0.71	0.94

No correlation as computed for promyelocytes, myelocytes, metamyelocytes, eosinophils and basophils due to poor correlation caused by too many zero values.

correlations. The correlation between RC and CC and the correlation between laboratory technicians is in the same order. Generally the correlations are higher for more frequent cell types and lower for less frequent cell types.

The figure shows the correlation coefficients between RC and CC and their confidence limits for the blood donors and the leukopenic patients. The confidence intervals normally found overlap for all cell types in the majority of healthy subjects and leukopenic patients.

Discussion

The results confirm the well known fact from quality control studies that in the differential blood counting the different cell types are determined with inconsistent accuracy [4]. This depends mainly on the frequency of the cell type. It may also, of course, depend on the difficulty of recognition of certain cells and on other random effects.

Routine peripheral blood films and films obtained from cell concentrates do not appear to correlate as well as expected. Sampling and human error introduced into the counting procedure reduces this correlation. The observed correlation would therefore be much less even if there were a true correlation between RC and CC in the order of 1.0. The correlation between the performance of the laboratory technicians is of the same order as the correlation between RC and CC. It can therefore be assumed that the true correlation between RC and CC which would be obtained by differentiating a large sample and by excluding human error would not be a great deal below 1.0.

The same results could be interpreted otherwise as follows: the statistical variation

Table II. Correlation coefficients (r) between RC and CC blood counts of 124 blood donors and between the counts of two laboratory technicians

Type of cells	Correlation coefficient between CC and RC			Correlation coefficient between the counts of two technicians of the same laboratory					
				CC			RC		
	r	confidence interval of r (p = 0.95)		r	confidence interval of r (p = 0.95)		r	confidence interval of r (p = 0.95)	
Segmented forms	0 70	0.59	0 78	0.77	0.69	0.83	0.65	0.53	0.74
Band forms	0 71	0.61	0.79	0.59	0.46	0.69	0.40	0.24	0.54
Lymphocytes	0.65	0.53	0.74	0.83	0 77	0.88	0.67	0.56	0.76
Monocytes	[illegible]	0.09	0.42	0.48	0.33	0.60	0.21	0.03	0.37
Eosinophils	0 71	0 61	0.79	0.66	0.55	0.75	0.42	0.26	0.55
Basophils	0.37	0.21	0.51	0.56	0.43	0 67	[illegible]	-0.16	0.19

No correlation was computed for myelocytes and metamyelocytes due to a poor distribution caused by too many zero values.

in RC films. However the different cell types do not equally concentrate. Table I shows that for example segmented forms concentrate more than monocytes. Table II shows the correlation coefficients between the RC values and the CC values and their confidence intervals for the films of the blood donors. Due to the absence of the myelocytes and metamyelocytes in many cases no satisfactory frequency distribution was found Therefore no correlation coefficients were computed for these two cell types. The correlation for the more frequent cell types (segmented forms and lymphocytes) is in the order of 0 7 Table II also indicates the correlation coefficients of the performance of the two laboratory technicians. The correlation between RC and CC is in the same order as the correlation between the two laboratory technicians. The correlation between the technicians is higher for CC than for RC for all cell types.

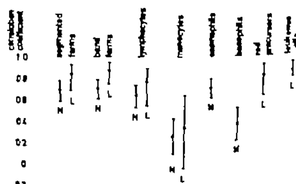


Fig. 1 Correlations between differential blood counts from RC and CC. N = blood donors; L = leukopenic patients vertical bars indicate correlation coefficient and confidence interval p = 0.95 of the correlation coefficient.

The same information is obtained from blood films made from patients with acute leukemia and severe leukopenia as shown in table III The frequency distribution of promyelocytes, myelocytes and metamyelocytes was insufficient for the computation of

Table III. Correlation coefficient ( ) in the differential blood counts from 10 patients with leukopenia (< 1,000 leukocytes/μl) obtained on 26 separate occasions

Type of cells	Correlation coefficient between CC and RC			Correlation coefficient between the counts of two technicians of the same laboratory					
				CC			RC		
		confidence interval of r (p = 0.95)			confidence interval of (p = 0.95)			confidence interval of (p = 0.95)	
Segmented forms	0.85	0.69	0.93	0.86	0.71	0.94	0.97	0.93	0.99
Band forms	0.88	0.75	0.95	0.56	0.25	0.78	0.6[illegible]	0.31	0.81
Lymphocytes	0.77	0.54	0.89	0.89	0.77	0.94	0.92	0.83	0.96
Monocytes	0.34	−0.05	0.64	0.34	−0.05	0.64	0.40	0.01	0.68
Red precursors	0.82	0.63	0.97	0.95	0.89	0.98	0.65	0.35	0.83
Leukemic cells	0.85	0.69	0.93	0.33	−0.07	0.64	0.86	0.71	0.94

No correlation was computed for promyelocytes, myelocytes, metamyelocytes, eosinophils and basophils due to poor correlation caused by too many zero values.

correlations. The correlation between RC and CC and the correlation between laboratory technicians is in the same order. Generally the correlations are higher for more frequent cell types and lower for less frequent cell types.

The figure shows the correlation coefficients between RC and CC and their confidence limits for the blood donors and the leukopenic patients. The confidence intervals normally found overlap for all cell types in the majority of healthy subjects and leukopenic patients.

Discussion

The results confirm the well-known fact from quality control studies that in the differential blood counting the different cell types are determined with inconsistent accuracy [4]. This depends mainly on the frequency of the cell type. It may also, of course, depend on the difficulty of recognition of certain cells and on other random effects.

Routine peripheral blood films and films obtained from cell concentrates do not appear to correlate as well as expected. Sampling and human error introduced into the counting procedure reduces this correlation. The observed correlation would therefore be much less even if there were a true correlation between RC and CC in the order of 1.0. The correlation between the performance of the laboratory technicians is of the same order as the correlation between RC and CC. It can therefore be assumed that the true correlation between RC and CC which would be obtained by differentiating a large sample and by excluding human error would not be a great deal below 1.0.

The same results could be interpreted otherwise as follows: the statistical variation

caused by changing from the routine peripheral blood method to the concentrated cell method is probably almost the same as that caused by allowing another laboratory technician to perform the differential blood count. So that the additional induced inaccuracy may still be acceptable by the time saving CC method. The greater absolute number of cells in the CC blood films suggests a greater reliability of the CC method as compared with the RC method, although this was not an important factor in the above outlined investigation as the same number of cells were counted using both methods.

Generally a better correlation was observed between the two laboratory technicians in the CC films than in the RC films from blood donors. This also suggests a high reliability of the CC method. The tendency was neither confirmed nor refuted by the results from leukopenic patients. A greater number of cells must be counted to increase the reliability. Counting a large number of cells takes much more time with the RC method than with the CC method.

Although the number of blood samples is rather small for the estimation of the correlation coefficient, the results are acceptable from the blood donors compared with the results from the leukopenic patients. It is therefore unlikely that a different cell concentrating phenomena is observed in leukopenic patients.

The time-saving CC method for performing differential blood counts from patients with severe leukopenia may be a worthwhile addition to routine laboratory methods. The additional variation introduced by the CC method is small and is probably compensated by greater reliability.

References

1 Cronkite, E. P., Fliedner, T. M., Bond, V. P., Rubini, J. R., Brecher, G. and Quastler, H. P.: Dynamics of hemopoietic proliferation in man and mice studied by H³-thymidine incorporation into DNA. Ann. N.Y. Acad. Sci. 77: 803–820 (1959).

2 Dacie, J. V. and Lewis, S. M.: Practical haematology; 4th ed. (Churchill, London 1970).

3 Grubbs, F. E.: Errors of measurement, precision, accuracy and the statistical comparison of measuring instruments. Technometrics 15: 53–66 (1973).

4 Rümke, C. L.: Die Zelldifferenzierung in Blutausstrichen. Variabilität der Ergebnisse. Triangel 4: 154–158 (1959).

5 Thöni, H.: Testing the difference between two coefficients of correlation. Biom. J. 19: 355–359 (1977).

Received: June 30, 1978
Accepted: August 18, 1978

Dr. med. Horst Pflieger, Department of Internal Medicine, Division of Haematology, University of Ulm, Steinhoevelstrasse 9, D-7900 Ulm (FRG)

Acta haemat. 61 155-160 (1979)

Duodenal Plasmacytoma

A Rare Primary Extramedullary Localization Simulating a Carcinoma

F Pentimone M Camici G Cini and D Levorato

Clinica Medica Generale II, Università di Pisa, Pisa

Key Words. Extramedullary plasmacytoma Multiple myeloma Monoclonal gammopathy

Abstract. A patient with duodenal plasmacytoma is described. The extramedullary localization preceded the complete humoural picture of multiple myeloma by 15 months. The previously reported cases of myeloma of primary gastrointestinal localization are reviewed.

Extramedullary plasmacytoma is a rare tumour: the primary localization is most often in the upper respiratory tract or the oral cavity [14] but it may occur in many different organs (spleen lymph nodes, liver lungs, genital organs, kidneys or thyroid) [1 4 13] The gastrointestinal tract is a relatively frequent primary site [2, 5 8 11 16] From a review by *Kindler* [9] of all the cases of extramedullary plasmacytoma reported in the literature the incidence of the gastrointestinal localization was 12.1 of a total of 207 cases which was second only to the incidence of the upper respiratory localization (66 1 /).

The site most often involved is the stomach. from the first case noted by *I adli and Popa* [17] in 1928, up to the present, 23 cases have been reported in the literature [6, 10, 15] The duodenum is only rarely involved. up to 1965 the only reported case was that of *Heffermann* [7] in 1947 *Derechin et al* [3] reported another case in 1970 which was complicated by obstructive jaundice. The rarity of extramedullary plasmacytomas with duodenal primary localization and the considerable diagnostic difficulties with which they are associated, leads us to report a new case which recently came to our attention.

Case Report

A 69-year-old man was in good health prior to February 1976, when he developed loss of appetite with gradual weight loss, nausea, intermittent vomiting, lassitude and vague epigastric pain with no relation to meals. His first hospitalization was in June 1976 Physical examination revealed modest liv enlargement with the edge extending 2 cm below the costal margin, smooth and nontender with slight pallor of the skin and mucosae. The laboratory examinations disclosed. ESR, 30 mm first hour 80 mm second hour CBC RBC 2,700,000 mm Hb 8.8 g/100 ml, MCV 90 μm



Fig. 1 X ray of upper gastrointestinal tract coarse filling defect at the levels of the superior flexure of the duodenal loop

WBC 3,000/mm³ neut. 68%, lymph 28%, mono 3%, eosin 1%, anisopoikilocytosis. Reticulocytes 3.5%, iron 30 μg/100 ml. Platelets. 125,000/mm³ Occult blood in stool. ++++

Barium meal and follow-through there was a coarse filling defect at the level of the superior flexure of the duodenal loop which appeared rigid, apparently due to an intraluminal mass with a tendency towards ulceration (fig. 1).

Gastroduodenoscopy the gastric mucosa was moderately hypertrophic. There was difficulty in passing the duodenal bulb beyond which a conspicuous and constant narrowing of the descending duodenum with asymmetric contractions and loss of normal pattern of mucosal folds was noted. The papilla was not visualized

A tentative diagnosis of duodenal neoplasm was made and the patient was transferred to surgery for operation.

Operative Findings A neoplasm, the size of a hen's egg, was found on the posterior wall of the superior duodenal flexure The neoplasm was excised at the submucosal level except for a small circular zone in which the excision was full thickness. A gastroenterostomy with duodenorrhaphy was done.

Histological examination. the intestinal wall was infiltrated by a dense mass of neoplastic cells with round nuclei, sparse chromatin, and small

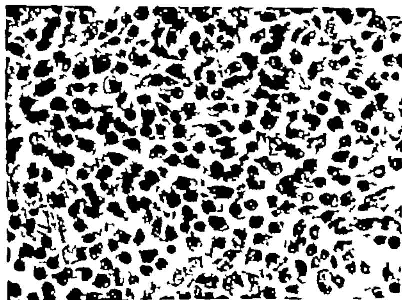


Fig. 2. Intestinal wall infiltrated by neoplastic cells with round nuclei, sparse chromatin and basophilic, small amounts of cytoplasm the nuclei have an eccentric position. HE×400.



Fig. 3. Lymph node: sheets of neoplastic cells with round nuclei, prominent cytoplasm, some mitoses with atypical figures. The nuclei were lightly stained, sometimes with clumped chromatin and with prominent nucleoli. HE × 400.

amounts of basophilic cytoplasm in which the nuclei occupied eccentric positions. Mitoses were frequent with modest number of atypical figures (fig 2). A histological diagnosis of duodenal plasmacytoma was made.

After surgery the patient underwent additional diagnostic examinations: bone marrow aspiration, search for Bence Jones proteins in the urine, blood alkaline phosphatase, total protein electrophoresis, immunoelectrophoresis and skeletal X-rays were within normal limits. The patient was discharged with diagnosis of duodenal plasmacytoma.

He returned to the clinic 3 months later after the appearance of two lymph nodes, the size of pigeon eggs. They were non-tender, freely moveable and located in the left lateral cervical region; one of the nodes was removed and examined. The normal architecture of the node was in large part obliterated by sheets of neoplastic cells with round nuclei, prominent cytoplasm, moderate anisocytosis and poikilocytosis and some mitoses with atypical figures. The nuclear characteristics varied but, in general, the nuclei were lightly stained, sometimes with clumped chromatin and with prominent nucleoli. A diagnosis of lymphoma of predominantly lymphoblastic type (fig. 3) was made.

months later in November 1976, the patient hospitalized for the third time. He was admitted with marked lassitude, weight loss, low grade fever, abdominal pain and widespread lymphadenopathy. ESR 45 mm first hour, 85 mm second hour. CBC: RBC 3,950,000/mm³, Hb 10 g/100 ml, MCV 80 μm³, WBC 3,000/mm³, neut. 60%, lymph 37%, mono 3%. Platelets: 793,000/mm³. Proteinemia, 7 g/100 ml.

Immunoelectrophoresis: IgG 3,100 mg/100 ml (normal range 570–1,700), IgA 356 mg/100 ml, IgM 102 mg/100 ml.

Blood marrow aspiration biopsy: absence of numerical or morphologic plasma cell anomalies. X-rays of flat bones: negative for osteolytic lesions.

Lymphography: In the lymphographic phase, there was a regular rise of contrast medium on the right, while on the left it was slowed and collaterals with dilated lymphatics were noted. In the adenographic phase, poverty of lymph nodes in the left iliac chains with irregular filling defects in the distal lymph nodes of the external iliac chain was noted.

Conclusion: involvement of the pelvic lymph nodes, especially on the left.

1 May 1977 the patient was hospitalized for the fourth time with thoracic herpes zoster, diffuse bone pain, lassitude, anaemia and oedema in the lower extremities. ESR 138 mm first hour, 14 mm second hour. γ-Glutamyl transpeptidase

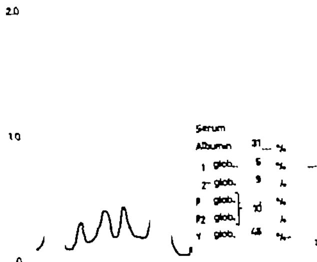


Fig. 4. Protein electrophoresis (May 1977)

23 mU/ml. Alkaline phosphatase 830 mU/ml. Total proteinemia. 8.3 g/100 ml Alb 31%, α -glob. 5 %, α_2 glob 9%, βglob 10%, γ-glob 45% (fig. 4)

Immunoelectrophoresis. prominent deformation of the IgG precipitation curve and of the k light chains: IgG paraproteinemia of type k (fig. 5). Search for Bence Jones proteinuria, negative.

CBC RBC 2,450 000/mm³ Hb 7 4 g/100 ml, MCV 90 μm³ WBC 2,100 mm³ neut. 89%, lymph 11 %. Platelets. 93,000/mm³ Bone marrow aspiration. of 1,000 marrow elements there were 38 plasma cells, most of which were morphologically atypical (fig. 6) the leuco-erythrogenic ratio was 0 23 Skeletal survey negative for myelomatoid lesions.

The patient's condition gradually deteriorated. a right pleural effusion developed, the oedema in the lower extremities increased and the platelet count fell to even lower levels (66,000/mm³) A severe gastrointestinal haemorrhage led to his demise on 20 6.77

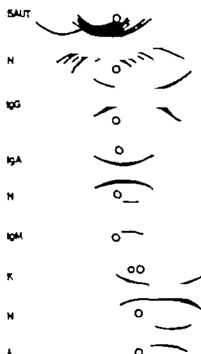


Fig. 5. Immunoelectrophoresis: IgG paraproteinemia of type k (May 1977).

Comment

The primary localization of a plasmacytoma in the duodenum is unusual as is indicated by the rarity of cases reported in the literature [12] This creates diagnostic difficulties which may be resolved only by histologic examination [18]

It is known on the other hand, that extramedullary plasmacytoma, wherever it is located, is characterized by an initial absence of skeletal lesions, absence of Bence-Jones proteinuria, and plasma cell anomalies in the marrow The typical manifestations of multiple myeloma may appear months or years after histological diagnosis of the localized form [4]

Lymph node involvement, rare in multiple myeloma is often observed in these cases [1] and sometimes is the basis for further diagnostic error This happened with our patient who was discharged from his third hospitalization with the diagnosis of lymphoma as suggested by extensive superficial and deep lymph node involvement both above and below the diaphragm In fact, the pres-



Fig. 6. Smear of bone marrow. Note the atypical plasma cells. Giemsa × 1,250.

ence of atypical cells which, even though destroying the normal architecture of the lymph node, did not clearly present as plasma cells, the persistent negativity of the humoural data and the improbability of a duodenal localization for a plasmacytoma, all led us to review critically the histological preparations of the excised duodenal mass. the scarcity of plasma cells and the greater incidence of lymphoma in the duodenum led to that diagnosis.

On the other hand, this interpretation did not exclude the possibility of duodenal plasmacytoma. in the literature [5–14] there are reported cases of extramedullary plasmacytoma tending towards lymphosarcomatous forms and, conversely, cases initially thought to be lymphomas which turned out to be plasmacytomas.

The evolution of the disease in our patient confirms the reports in the literature of the unpredictable evolution of extramedullary plasmacytoma, at times leading to a multiple myeloma or mesenchymal neoplasia and at times behaving like a relatively benign neoplasm.

The confirmation of the histological diagnosis of extramedullary plasmacytoma and the evaluation of its malignant potential is given only by its clinical evolution and the biological behaviour of the lesion [4].

The time that intervened between the primary duodenal localization and the diffuse form, in our case, was 15 months; the duodenal involvement was thus only a temporary extramedullary localization of a multiple myeloma.

References

1 Atti della Soc. It. Med. Int. 7° Congresso Montecatini Terme: Il mieloma multiplo (Pozzi, Roma 1971).
Bellantuono, N. et al.: Solitary plasmacytomas of the small intestine. Rass. int. Clin. Terap. 51: 936–947 (1971).
3 Derechin, M. M., et al.: Extraosseous plasmacytomas causing extrahepatic cholestasis and cardiac tamponade. Scand. J. Haematol. 7: 318 (1970).
4 Dolin, S. and Dewar, J. P.: Extramedullary plasmacytoma. Am. J. Path. 320: 83–100 (1956).

2.0

1.0

0

Serum
Albumin 31 %
α_1 glob. 5 %
α_2 glob. 9 %
β glob. }
β_2 glob. } 10 %
γ glob. 45 %

Fig. 4 Protein electrophoresis (May 1977)

23 mU/ml. Alkaline phosphatase 830 mU/ml. Total proteinemia, 8.3 g/100 ml. Alb. 31 %, α-glob. 5 %, α glob. 9%, β-glob. 10%, γ-glob. 45% (fig. 4).

Immunoelectrophoresis prominent deformation of the IgG precipitation curve and of the k light chains: IgG paraproteinemia of type k (fig. 5). Search for Bence Jones proteinuria negative.

CBC RBC 2,450,000/mm³ Hb 7.4 g/100 ml, MCV 90 m WBC 2,100 mm³ neut 89%, lymph 11 %. Platelets, 93 000/mm Bone marrow aspiration of 1,000 marrow elements there were 38 plasma cells, most of which were morphologically atypical (fig. 6) the leuco-erythrogenic ratio was 0.23 Skeletal survey negative for myelomatoid lesions.

The patient's condition gradually deteriorated a right pleural effusion developed, the oedema in the lower extremities increased and the platelet count fell to even lower levels (66,000/mm³) A severe gastrointestinal haemorrhage led to his demise on 20.6.77

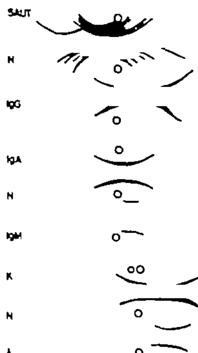


Fig. 5. Immunoelectrophoresis: IgG paraproteinemia of type k (May 1977)

Comment

The primary localization of a plasmacytoma in the duodenum is unusual as is indicated by the rarity of cases reported in the literature [12] This creates diagnostic difficulties which may be resolved only by histologic examination [18]

It is known on the other hand, that extramedullary plasmacytoma wherever it is located, is characterized by an initial absence of skeletal lesions, absence of Bence-Jones proteinuria, and plasma cell anomalies in the marrow The typical manifestations of multiple myeloma may appear months or years after histological diagnosis of the localized form [4]

Lymph node involvement rare in multiple myeloma, is often observed in these cases [1] and sometimes is the basis for further diagnostic error This happened with our patient who was discharged from his third hospitalization with the diagnosis of lymphoma as suggested by extensive superficial and deep lymph node involvement both above and below the diaphragm In fact the pres



Fig. 6. Smear of bone marrow. Note the atypical plasma cells. Giemsa × 1,250

ence of atypical cells which, even though destroying the normal architecture of the lymph node, did not clearly present as plasma cells, the persistent negativity of the humoural data and the improbability of a duodenal localization for a plasmacytoma, all led us to review critically the histological preparations of the excised duodenal mass. the scarcity of plasma cells and the greater incidence of lymphoma in the duodenum led to that diagnosis.

On the other hand, this interpretation did not exclude the possibility of duodenal plasmacytoma. in the literature [5 14] there are reported cases of extramedullary plasmacytoma tending towards lymphosarcomatous forms and, conversely cases initially thought to be lymphomas which turned out to be plasmacytomas.

The evolution of the disease in our patient confirms the reports in the literature of the unpredictable evolution of extramedullary plasmacytoma, at times leading to a multiple myeloma or mesenchymal neoplasia and at times behaving like a relatively benign neoplasm.

The confirmation of the histological diagnosis of extramedullary plasmacytoma and the evaluation of its malignant potential is given only by its clinical evolution and the biological behaviour of the lesion [4]

The time that intervened between the primary duodenal localization and the diffuse form, in our case, was 15 months. the duodenal involvement was thus only a temporary extramedullary localization of a multiple myeloma.

References

1 Atti della Soc It. Med. Int. 72 Congresso Montecatini Terme: Il mieloma multiplo (Pozzi, Roma 1971)

2 Bellantuono, N et al Solitary plasmacytomas of the small intestine. Rass. Int. Clin. Terap 51 936–947 (1971).

3 Derwahm, M M et al. Extraosseous plasmacytomas causing extrahepatic cholestasis and cardiac tamponade Scand J Haematol. 7 318 (1970)

4 Dolin, S. and Dewar J P Extramedullary plasmacytoma. Am. J Path. 320 83–100 (1956).

5 Elias, E. G et al Extraosseous multiple myeloma. A cause of intestinal obstruction. Ann. Surg. *170* 857–861 (1969).
6 Fontana, G et al Su un caso di plasmacitoma con localizzazione gastrica. Riv Patol. Clin .2 1501 (1967)
7 Haffermann, A.. Plasmacytoma of pancreas and duodenum causing acute intestinal obstruction. Lancet *I.* 910 (1947).
8 Ingegno A P Plasmacytoma of the gastrointestinal tract, report of a case involving the jejunum and review of the literature. Gastroenterology *26.* 89 102 (1954)
9 Kindler U Sul plasmocitoma extraosseo. Med. Ted. *I* 444 (1965)
10 Melletti G e Russo, C Rara osservazione di plasmocitoma extramidollare a sede gastrica. Rass. int Clin. Terap *59* 144 –1450 (1969).
11 Merritt, J W jr Plasmacytoma of the gastrointestinal tract. Ann. Surg. *14* 881–894 (1955)
12 Polli E. The duodenum in systemic disease. Minerva med. *63* 3892–3895 (1972)
13 Polli, E. La leucemia biologia e clinica (Pic c n, Padova 1967).
14 Rawson, A. J et al Plasma-cell tumors of the upper respiratory tract a clinical-pathologic study with emphasis on criteria for histologic diagnosis. Am. J Path. *26* 445–461 (1950).
15 Remigio, P A., et al. Extramedullary plasmacytoma of stomach. Cancer *27* 562 568 (1971).
16 Sharma, K. D and Shrivastav J D Extramedullary plasmacytoma of gastrointestinal tract. Archs Path. *71* 229 233 (1961).
17 Vasiliu, T et Popa R.. Formes gastro-intestinales des tumeurs dites plasmacytomas. C. r Séanc Soc. Biol. *98* 738 (19.8).
18 Zivielio, M et al.. Radiologic diagnosis of primary malignant duodenal neoplasms. Rass. int. Clin. Terap *52* 487–500 (1972).

Received November 3 1977
Accepted. June 15, 1978

F Pentimone, Clinica Medica Generale II, Università di Pisa Pisa (Italy)

Acta haemat. 61 161–167 (1979)

Platelet Aggregation and Retinal Microangiopathy in Diabetes and Hypertension

P. K. Khosla, M. Mahabaleswara, H. K. Tiwari and A. K. Saraya

Dr. Rajendra Prasad Centre for Ophthalmic Sciences, and Haematology Unit, Department of Pathology, All India Institute of Medical Sciences, New Delhi

Key Words. Platelet function · Retinal microangiopathy · Diabetic retinopathy · Hypertensive retinopathy

Abstract. Platelet aggregation studies were done in 10 cases of diabetes with and without retinopathy and 7 cases of hypertensive retinopathy with adenosine diphosphate (ADP) and adrenaline (ADR). It was observed that the rate and degree of aggregation was significantly increased in the retinopathy group both with ADP and ADR. A significant alteration in the latent period was found with ADP whereas no such change was found with ADR. It is proposed that the increased platelet reactivity in retinal microangiopathy could be due to different mechanisms. Increased sensitivity of the platelets to ADP resulting in the rapid inductive phase of aggregation together with accelerated intrinsic ADP release in diabetic retinopathy may cause hyperaggregation of platelets. Hyperaggregation in hypertensive retinopathy, however, may occur due to accelerated ADP release only. Platelet metabolism supporting the release reaction is altered in both.

Introduction

Increased platelet aggregation in diabetic patients with retinopathy has been reported in several studies [1–4]. Hyperaggregation of platelets, however, has also been observed in vasculopathies in the absence of diabetes [5] as well as in prediabetes, latent diabetes and diabetes without vasculopathies [6] and in hypertensive retinopathy [7].

The mechanism of aggregation abnormality, however, is not understood. In the present study we have determined the changes in the total events, as observed on an aggregometer, occurring in platelets on addition of adenosine diphosphate (ADP) and adrenaline (ADR).

Hyperaggregation of platelets has been found in patients with diabetic retinopathy as well as in hypertensive retinopathy. It has been proposed that in diabetic retinopathy a rapid inductive phase of ADP-induced platelet aggregation and accelerated intrin-

sic ADP release results in hyperaggregation. The latter alone may be operative in hyper tensive retinopathy

Materials and Methods

Patients with diabetes with or without retinopathy and patients with *hypertensive retinopathy* attending the outpatients department were taken for study The initial workup included a detailed clinical examination and laboratory investigation to assess the clinical grading of retinopathy and biochemical severity of the diabetes. Serum cholesterol along with fasting and postprandial blood sugar levels were determined by standard tech niques. Routine urine examination, and blood urea estimation when indicated were carried out. Fluorescent angiography was done in each patient.

Three groups of patients were studied (table I). Group I consisted of 10 patients of diabetes without retinopathy The average age of the patients was 54 years, the mean duration of diabetes being 5 72 years. Group II consisted of 10 patients of diabetes with clinical evidence of retinopathy The average age was 51 9 years and the mean duration of diabetes was 7 4 years. Group III 7 patients in this group had hypertension and advanced retinopathy (grade III and IV) The average age of these patients was 4? years and the mean duration of hypertension 5.6 years. Normal controls were amongst the healthy laboratory workers and doc tors without diabetes and hypertension. None of these had taken any drugs known to affect platelet aggregation for 10 days prior to study In each of these patients platelet aggregation was studied along with the controls matched for age and sex by the method of *Born* [8] using a chronolog aggregometer *The change in optical density* and the increment rate of aggregation as computed by the differentiometer connected to the aggregometer were studied with ADP and ADR.

Platelet aggregation was studied with 0.25 and 0.5 μg of ADP and ADR in 0.02 ml imidazole buffer pH 7.2/0 4 ml of platelet rich plasma (platelet count 300,000/μl) In group III, however it was studied with 0.5 μg ADR only and was evaluated by the Student's t test. Other graphic recordings were analyzed statistically with respect to latent period (LP) and maximum aggregation as well as its rate and duration by analysis of variance and Duncan s multiple range test. These par ameters have been recommended by *Barbui and Battista* [9] to completely assess the platelet reac tivity to aggregating agents and are defined below

Latent Period. It was calculated as the time interval between the addition of ADP or ADR and the beginning of primary aggregation curve.

Rate of Aggregation. It relates to the maxi-

Table I. Age and duration of disease process of patients of groups I II and III

Group I diabetes		Group II diabetic retinopathy		Group III hypertensive retinopathy	
age/sex	duration years	age/sex	duration years	age/sex	duration years
60/F	2/12	58/F	16	38/F	8
60/M	10	36/F	8	63/M	⌐1
70/M	10	50/M	11	58/M	5
75/F	4	44/M	11	49/M	1
47/M	2	65/M	2	22/M	1
62/M	12	52/M	4/12	27/M	⌐/12
52/M	4	62/M	2	30/M	4
18/M	7	55/M	7		
40/M	6	38/M	7		
62/M	2	59/F	10		

mum speed of aggregation and is read on differentiometer recording and expressed as number of divisions/min.

Degree of Aggregation. It relates to the maximal change in light transmission and is expressed as a percentage. 100% was adjusted against platelet-poor plasma.

Duration of Aggregation. It was measured as time interval between the initiation of aggregation on ADP or ADR addition and the completion as

Table II. Range, mean values and SE of primary platelet aggregation with ADP in normal subjects and patients of groups I–III analyzed by analysis of variance and Duncan's multiple range test at the 5% level

	Aggregation[1]						Latent period, sec	
	rate (number of division/min)		degree, %		duration, sec			
Dose of ADP	0.25 µg	0.50 µg	0.25 µg	0.50 µg	0.25 µg	0.50 µg	0.25 µg	0.50 µg
Normal (n = 19)								
Mean	23.44	28.85	31.00	43.16	107.05	134.74	4.53	3.63
Range	13.00 – 35.75	13.00 – 42.75	14.00 – 50.00	19.00 – 60.00	30–210	45–180	2–6	2–6
SE	± 1.73	± 1.73	± 2.98	± 2.98	± 12.51	± 12.51	± 0.30	± 0.30
Group I Diabetes (= 10)								
Mean	22.39	31.15	29.30	42.00	90.30	106.70	5.20	4.50
Range	8.12 – 29.25	22.75 – 39.00	8.00 – 56.00	22.00 – 60.00	33–210	45–210	3–9	3–9
SE[2]	± 2.39	± 2.39	± 4.11	± 4.11	± 17.23	± 17.23	± 0.41	± 0.41
Group II Diabetes with retinopathy (= 10)								
Mean	29.61	39.19	45.70	58.60	134.00	132.50	3.40	3.10
Range	22.75 – 35.50	26.00 – 65.25	26–56	34–78	50–230	60–180	2 5	2 5
SE[2]	± 2.39	± 2.39	± 4.11	± 4.11	± 17.23	± 17.23	± 0.41	± 0.41
Group III Retinopathy with hypertension (n = 7)								
Mean	28.83	37.61	38.57	51.86	102.29	152.28	5.14	3.71
Range	19.50 – 42.25	29.25 – 42.25	22–60	36–66	60–150	110–186	5–6	3–4
SE	± 2.86	± 2.86	± 4.91	± 4.91	± 20.60	± 20.60	± 0.49	± 0.49
Contrast between doses	$p < 0.01$		$p < 0.01$		not significant		$p < 0.01$	
Contrast between groups	*(II and III) vs (N and I)		*(I and N) vs (II) *(I and III) vs ***(N and III)		not significant		*(I and II) vs **(N and I) ***(N and II) vs **(II and III)	

[1] Maximum height of differentiometer 3.25 (calibration factor).
[2] From analysis of variance.
[3] With both doses of ADP **0.25 µg of ADP ***0.5 µg of ADP

observed by drawing a perpendicular to the most flattened part of the curve.

Results

The discriminating parameters were rate and degree of aggregation and the LP The response was dose related both with ADP and ADR.

In diabetes (group I) the rate and degree of aggregation with ADP or ADR were similar to the normal subjects but the LP was significantly higher with ADP alone (table II III) The latter was statistically significant only with 0 25 μg ADP but was highly suggestive with 0.5 μg

Disaggregation following primary aggregation with 0.25 μg of ADP was seen in 60% patients in both the groups.

Table III. Range, mean values and SE of primary platelet aggregation with adrenaline in normal subjects and patients of groups I and II analyzed by analysis of variance and Duncan s multiple range rest at the 5 % level

	Aggregation[1]						Latent period, sec	
	rate (number of divisions/min)		degree %		duration, sec			
Dose of adrenaline	0.25 μg	0.50 μg	0.25 μg	0.50 μg	0.25 μg	0.50 μg	0.25 μg	0.50 μg
Normal (n = 8)								
Mean	6.70	8.25	8.50	11 62	71.00	77 75	4.37	3.12
Range	4 87 − 11.57	4.50 − 19.25	5.00 − 17 00	6.00 − [illegible]2.00	45–90	30–135	2 9	2 5
SE	± 1 45	± 1 45	± 2.34	± 2.34	± 11.24	± 11.[illegible]4	± 0 64	± 0.64
Group I (n = 4)								
Mean	6.50	9 65	9 65	15 00	75.00	67.50	5 70	4 00
Range	4 87 − 8 12	4.87 − 13.00	6.00 − 14.00	6.00 − 18.00	60–90	60–90	3–9	3–5
SE	± 2.05	± 2.05	± 3.31	± 3.31	± 15 89	± 15.89	± 0.90	± 0.90
Group II (n = 4)								
Mean	9 64	17.50	17.50	30.50	78.00	102.50	5 00	3.50
Range	4 87 − 16.[illegible]5	11.37 − 26.00	10.00 − 26.00	20.00 − 50.00	60–90	60–200	3–6	2–6
SE[2]	± 2.05	± 2.05	± 3.31	± 3.31	± 15.89	± 15 89	± 0.90	± 0.90
Contrast between doses	p < 0.05		p < 0.01		not significant		p < 0.05	
Contrast between groups	(N and I) vs (II)		(N and I) vs (II)		not significant		not significant	

Figures in parentheses refer to number of observations analyzed by analysis of variance. Group III was not analyzed by analysis of variance.

1 Maximum height of differentiometer charting × 3.25 (calibration factor).

2 From analysis of variance.
With both doses adrenaline *0.5 μg of adrenaline.

In patients with diabetic retinopathy (*group II*), *differences* were noted in these *parameters when compared with diabetes* alone (table II, III). Rate and degree of aggregation with ADP was higher in these patients with both the doses. However with ADR significant differences were seen in rate with 0.5 μg, but in degree with both the concentrations. Similar findings were also noted when compared with normal subjects. The latent period, however was significantly shortened with ADP as compared to in *diabetes with both concentrations* and in normal subjects with 0.5 μg.

In hypertensive retinopathy (group III) rate and degree of aggregation was higher than normal subjects as well as in diabetes alone with ADP (table II). The latent period was not significantly altered as compared to normal and diabetic subjects, but was longer than those with diabetic retinopathy.

No difference with ADR was noticed in the latent period amongst these groups. *However, the rate of aggregation* with 0.5 μg ADR in group III was higher (mean 16.28 ± 6.60) as compared to normal subjects (mean 10.42 ± 6.02). This difference was significant ($t = 2.12$, $p < 0.05$). Degree of aggregation in these was also higher (mean 32.42 ± 14.52%) but not significant when compared with other groups or normal subjects. Disaggregation in patients with retinopathy in association either with *diabetes or hypertension was not seen with* 0.25 μg ADP.

Discussion

Parameters of the platelet functions in normal subjects and in those with diabetes without retinopathy did not show any difference except in LP which was longer in the latter. Patients with diabetes and retinopathy on the other hand, *had shorter* LP faster rate and greater degree of platelet aggregation. Patients with hypertension associated with retinopathy also had faster rate and greater degree of aggregation but the LP was longer than in patients with diabetic retinopathy. Both in diabetic and hypertensive retinopathy the disaggregation was not seen with 0.25 μg ADP and a prolonged uninterrupted aggregation occurred. Dose-related correlation with ADP and ADR was seen within each group of these patients and in normal subject. Presence of diabetes thus alone was not associated with hyperaggregation of platelets and these studies do not support the findings of *Sagel et al* [6]. A subsequent study of the platelet reactivity in diabetic patients did not reveal the altered platelet reactions [10].

Increased platelet aggregation to ADP was seen both with shorter LP (diabetic retinopathy) and with longer LP (hypertensive retinopathy), and a longer LP was also seen with normal aggregation (diabetic patients). It appears that hyperaggregation can be caused by mechanisms other than rapid induction phase (shorter LP) but the latter could be an added factor. It has been postulated that a specific receptor on platelet membrane binds ADP [11] and that the binding protein may be related to actomyosin *present in platelets* [12]. *Reassociation* of dissociated actin and myosin on adjacent platelets have been said to make the intermolecular bridges and thus cause platelet aggregation [13]. Reduction of actomyosin glycoprotein [13, 14] and surface-bound fibrinogen [15, 16] have been incriminated to cause failure of ADP-induced platelet aggregation in Glanzmann's thrombasthenia.

Absence of aggregation has also been noted in glutathione peroxidase deficiency [17] Increase in these substances or in active re ceptor sites on platelet membrane resulting in hyperaggregation remains to be explored

A continued platelet aggregation uninter rupted by disaggregation seen with retinopa thy both in diabetic and hypertensive pa tients suggests that a quicker and greater re lease of ADP occurred in these patients This could be the predominant factor in causing hyperaggregation. Reactivity of pla telets depends upon its energy metabolism. It has been shown that an increasing amount of ATP is required for progressive platelet reactions and a high level of ATP is required to support release of endogenous ADP although ATP is not consumed dur ing aggregation [18] The role of prostag landings in mediating the relase of intrinsic ADP has been emphasized recently Labile cyclic endoperoxide, a precursor of prostaglandin (PG) E_2 and F_{2a} [19–22] can directly induce ADP release. This endoperoxide has been referred to as labile aggregation stimulating substance (LASS) [21] Prostaglandin E_2 potentiates LASS-induced release reac tion [12] leading to increased intrinsic ADP release responsible for secondary aggrega tion.

The present observations do not permit us to completely define the pathogenetic pathways of hyperaggregation seen in diabetic and hypertensive retinopathy It is proposed that retinopathy and altered platelet function may be etiopathogenetically relat ed. Increased aggregation of platelets is caused by metabolic changes leading to ac celerated intrinsic ADP release. Changes in ADP membrane receptors resulting in shorter LP could be an added factor Prima ry metabolic changes leading to accelerated intrinsic ADP release may alone cause hy peraggregation of platelets in hypertensive retinopathy but in diabetic retinopathy both these mechanisms may be involved.

References

1 Heath, H., Brigden, W D Canever J V Pollock, J., Hunter P R. Kelsey J and Bloom, A. Platelet adhesiveness and aggregation in relation to diabetic retinopathy Diabetologica 7 308–315 (1971).

2 Kwaan, H C. Colwell, J A. Cruz, S. Suwanwela, N and Dobbie, J G Increased platelet aggregation in diabetes mellitus. J Lab. clin. Med. 80 236–245 (1972).

3 Passa, P., Bensoussan, D., Levy-Toledano, S. Caen, J et Canivet, J.. Etude de l'agrégation plaquettaire au cours de la rétinopathie diabétique. Influence de l'hypophysectomie. Atherosclerosis 19 277–285 (1974)

4 Bensoussan, D Levy-Toledano, S., Passa, P., Caen, J and Canivet, J Platelet hyperaggregation and increased plasma level of Von Willebrand factor in diabetics and retinopathy Diabetologica 11 307–312 (1975).

5 Leone, G., Bizzi, B., Accoria, F and Boni, P Functional aspects of platelets in diabetes mellitus in Caprino and Rossi, Platelet aggregation and drugs, pp. 49–61 (Academic Press, New York 1974).

6 Sagel J Colwell, J A. Crook, L., and Laimins, M. Increased platelet aggregation in early diabetes mellitus. Ann. Intern. Med. 82 733–738 (1975).

7 Kok, D A. and Watson, P G Platelet aggregation in retinal vascular disease in vision and circulation. Proc. 3rd William MacKenzie Memorial Symp 1974 pp. 303–314 (Henry Kimpton, London 1976).

8 Born, G V R.. Aggregation of blood platelets by adenosine-diphosphate and its reversal. Nature, Lond. 194 927–929 (1962).

9 Barbui, L e Battista, R.. Studio quantitativo dell aggregazione piastrinica da ADP e da adrenalina. Mediante l'aggregometro di Mustard. Ric. clin. Lab. 2 299–312 (1972)

10 Colwell, J A.; Halushka, P V.; Sarji, K., Levend, J.; Sagel, J and Nair R. M. G. Altered platelet function in diabetes mellitus. Diabetes 25 suppl. 2, pp. 826–831 (1976).
11 Nachman, R. L. and Ferris, B. Binding of adenosine diphosphate by isolated membranes from human platelets. J biol. Chem. 249: 704–710 (1974).
12 Weiss, H. J Platelet physiology and abnormalities of platelet functions. New Engl. J Med. 293. 531 541 (1975).
13 Booyse, F Kisieleski, D.; Seeler R., and Rafelson, M., jr Possible thrombosthenin defect in Glanzmann's thrombasthenia. Blood 39 377–381 (1972).
14 Nurden, A. T and Caen, J P An abnormal platelet glycoprotein pattern in three cases of Glanzmann thrombasthenia. Br. J. Haemat. 28. 253–262 (1974)
15 Weiss, H. J and Kochwa, S. Studies of platelet function and protein in 3 patients with Glanzmann's thrombasthenia. J Lab. clin. Med. 71 153–165 (1968).
16 Caen, J P Glanzmann thrombasthenia; in Brien, Clinics in haematology vol. 1, pp. 383–392 (Saunders, Philadelphia 1972).
17 Karpatkin, S. and Weiss, H. J Deficiency of glutathione peroxidase associated with high levels of reduced glutathione in Glanzmann's thrombasthenia. New Engl. J Med. 287 1062 1066 (1972).
18 Holmsen, H., Setkowsky C. A., and Day H. J Effects of antimycin and 2-deoxyglucose on adenine nucleotide in human platelets. Role of metabolic adenosine tri-phosphate in primary aggregation, secondary aggregation and shape changes in platelets. Biochem. J 144 385–396 (1974).
19 Smith, J B. and Macfarlane, D. E. Platelets; in Ramwell, The prostaglandins, vol. 2, pp. 293–343 (Plenum Press, New York 1974).
20 Hamberg, M., Svensson, J Wakabayashi, T and Samuelsson, B. Isolation and structure of two prostaglandin endoperoxides that cause platelet aggregation. Proc. natn. Acad. Sci. USA 71 345–349 (1974).
21 Willis, A. L., Vane, F M., Kuhn, D. C. Scott, C. G and Petrin, M.: An endoperoxide aggregator (LASS), formed platelets in response to thrombotic stimuli. Purification, identification and unique biological significance. Prostaglandins 8 453–507 (1974).
22 Smith, J B., Ingerman, C., Kocsis, J J and Silver M. J Formation of an intermediat in prostaglandin biosynthesis and its association with the platelet release reaction. J clin. Invest. 53. 1468–1472 (1974).

Received: March 13, 1978
Accepted: July 31, 1978

Dr A. K. Saraya, Department of Pathology
All-India Institute of Medical Sciences,
New Delhi – 110016 (India)

Absence of aggregation has also been noted in glutathione peroxidase deficiency [17] Increase in these substances or in active receptor sites on platelet membrane resulting in hyperaggregation remains to be explored.

A continued platelet aggregation uninterrupted by disaggregation seen with retinopathy both in diabetic and hypertensive patients suggests that a quicker and greater release of ADP occurred in these patients This could be the predominant factor in causing hyperaggregation. Reactivity of platelets depends upon its energy metabolism It has been shown that an increasing amount of ATP is required for progressive platelet reactions and a high level of ATP is required to support release of endogenous ADP although ATP is not consumed during aggregation [18] The role of prostaglandings in mediating the relase of intrinsic ADP has been emphasized recently Labile cyclic endoperoxide, a precursor of prostaglandin (PG) E_2 and $F_{2\alpha}$ [19–22] can directly induce ADP release This endoperoxide has been referred to as labile aggregation stimulating substance (LASS) [21] Prostaglandin E_2 potentiates LASS-induced release reaction [12] leading to increased intrinsic ADP release responsible for secondary aggregation.

The present observations do not permit us to completely define the pathogenetic pathways of hyperaggregation seen in diabetic and hypertensive retinopathy It is proposed that retinopathy and altered platelet function may be etiopathogenetically related. Increased aggregation of platelets is caused by metabolic changes leading to accelerated intrinsic ADP release. Changes in ADP membrane receptors resulting in shorter LP could be an added factor Primary metabolic changes leading to accelerated intrinsic ADP release may alone cause hyperaggregation of platelets in hypertensive retinopathy but in diabetic retinopathy both these mechanisms may be involved.

References

1 Heath, H., Brigden, W D., Canevar J V., Pollock, J Hunter P R., Kelsey J and Bloom, A.. Platelet adhesiveness and aggregation in relation to diabetic retinopathy Diabetologica *7* 308–315 (1971).

2 Kwaan, H. C., Colwell, J A. Cruz, S. Suwanwela, N., and Dobbie, J G Increased platelet aggregation in diabetes mellitus. J Lab. clin. Med. *80* 236–245 (1972).

3 Passa, P., Bensoussan, D., Levy Toledano, S., Caen, J et Canivet, J Etude de l'agrégation plaquettaire au cours de la rétinopathie diabétique. Influence de l'hypophysectomie. Atherosclerosis *19* 277–285 (1974).

4 Bensoussan, D Levy-Toledano, S. Passa, P., Caen, J and Canivet, J.. Platelet hyperaggregation and increased plasma level of Von Willebrand factor in diabetics and retinopathy Diabetologica *11* 307–312 (1975).

5 Leone, G., Bizzi, B., Accoria, F., and Boni, P Functional aspects of platelets in diabetes mellitus; in Caprino and Rossi, Platelet aggregation and drugs, pp. 49–61 (Academic Press, New York 1974).

6 Sagel, J Colwell, J A. Crook, L., and Lahmins, M.. Increased platelet aggregation in early diabetes mellitus. Ann. intern. Med. *82* 733–738 (1975).

7 Kok, D A. and Watson, P G Platelet aggregation in retinal vascular disease in vision and circulation. Proc. 3rd William Mackenzie Memorial Symp., 1974 pp. 303–314 (Henry Kimpton, London 1976)

8 Born, G V R.. Aggregation of blood platelets by adenosine-diphosphate and its reversal. Nature, Lond. *194* 927–929 (1962).

9 Barbui, L e Battista, R. Studio quantitativo dell'aggregazione piastrinica da ADP e da adrenalina. Mediante l'aggregometro di Mustard. Ric. clin. Lab. 2. 299–312 (1972).

[10] The finding of a case of CMML in which PK deficiency could be shown to be congenital makes this report warranted.

Case Report

The patient, 72-year-old woman, was admitted to the University Hospital of Barcelona for treatment of fever which persisted for 2 months. On physical examination the patient showed left supraclavicular adenopathy painful hepatomegaly and spleen enlargement. Hematological examination showed her to have severe anemia with leukocytosis (table I).

Blood smears showed intense anisopoikilocytosis with abundant peroxidase-negative monocytoid leukocytes which were positive to naphthol-AS-D-acetate esterase (NASDA) stain. The positivity to NASDA reaction disappeared almost completely in the presence of sodium fluoride (NaF) According to the morphological and cytochemical criteria recommended by the FAB cooperative group for the classification of acute leukemias [3], it can be assumed that the vast majority of the atypical leukocytes found in the peripheral blood smear was monocytes.

Bone marrow examination showed normal myeloerythroid ratio, 15% of peroxidase-positive blastic cells and 35% of monocytoid cells with similar morphological and cytochemical character istics to those observed in peripheral blood. Mar row iron content was normal with no pathological sideroblasts ('ring sideroblasts'). Ultrastructural studies of the patient's bone marrow cells demonstrated dyshemopoietic features in all blood cell precursors (erythrocytic, granulopoietic and me gakaryocytic) and especially in erythroblasts, some of which presented nuclear 'clefts' and intramitochondrial iron content. Bone marrow biopsy showed diffuse invasion of blastic cells with par tial preservation of cellularity and architecture; no fibrosis could be appreciated. The patients' karyotype was normal.

Material and Methods

Routine hematologic studies were performed by standard methods. Plasmatic muramidase was determined by the method of *Parry et al.* [35]. Electrophoretic studies of hemoglobin were car ried out at pH 8.6 on cellulose acetate strips. Alkali-resistant hemoglobin (HbF) was measured by the Singer method [41] and agglutination of the patients' red cell with anti-I serum was quantified according to methods described elsewhere [28].

Erythrocyte enzyme determination was carried out by methods previously described [6, 47]. Acetylcholinesterase (AChE) activity of erythrocytes was studied from whole blood sample according to *Browdson and Watts* [11]. After drawing, erythrocytes were separated from leukocytes and platelets by filtration through DEAE-cellulose Sephadex column [33]. Substrates and enzymes employed in the enzymatic reactions came from Boehringer Mannheim, and Sigma, St. Louis, Mo. Readings at 340 nm were mad with Beckmann DBGT spectrophotometer linked to a recorder. Red cell reduced glutathione (GSH) content and its stability in the presence of acetylphenylhydrazine (APH) was determined by the method of *Beutler et al.* [4]. ATP content was measured according to *Mbaskwi et al.* [30] and 2,3-diphosphoglycerate (2,3-DPG) was determined by the method of Sigma [40]. Erythrocytes were separated into young and old populations with the microhematocrit method of *Herz and Kaplan* [23].

Results

Coomb s test and acidified serum lysis tests were repeatedly negative. Serum bilirubin, iron content, vitamin B_{12}, folic acid and red cell folates were found within normal limits (table I) The patient's plasmatic mur amidase was strikingly increased to 350 µg/ml (normal range. 4 6–9.5).

Electrophoretic studies of hemoglobin showed a decrease in the HbA_2 fraction to 1 / (normal range 2.5–3 /) on the contrary alkali-resistant HbF was increased to 2.8°/ (normal range 0–2°). Agglutination of the patient's red cells with anti-I serum was increased to a titer of 1/16 (normal cord blood. 1/32, normal adult red cells.

Case Report

Acta haemat. *61* 168–174 (1979)

Chronic Myelomonocytic Leukemia Associated with Hereditary Pyruvate Kinase Deficiency and Multiple Acquired Erythrocyte Abnormalities

Joan Lluís Vives-Corrons, Lourdes Florensa, Josep Muncunill, Benet Nomdedeu and Ciril Rozman[1]

Postgraduate School of Hematology 'Farreras Valentí' and Central Hematology Laboratory Hospital Clínico y Provincial, University of Barcelona, Barcelona

Key Words. Chronic myelomonocytic leukemia · Pyruvate kinase deficiency · Acquired erythroenzymopathies

Abstract. A congenital erythrocyte pyruvate kinase (PK) deficiency was found in a 72 year-old female patient with chronic myelomonocytic leukemia (CMML). Erythrocyte PK deficiency was associated with an increase in the activity of hexokinase, 6-phosphogluconate dehydrogenase and glutathione peroxidase in erythrocytes as well as a decrease in acetylcholinesterase, glutathione reductase and glucosephosphate isomerase activities. The enzymatic abnormalities were accompanied by alterations in hemoglobin and in i antigen content of erythrocyte membrane. In addition, bone marrow ultrastructural studies showed dyshemopoietic changes in all blood cell lines and especially in erythroblasts. The present findings confirm the close relationship between CMML and acquired dyserythropoietic syndromes and constitute a new observation of the infrequent association of hereditary erythrocyte enzymopathies and leukemia. A survey of the literature is presented.

Introduction

Chronic myelomonocytic leukemia (CMML) in adults is a form of an acquired dyshemopoietic syndrome characterized by anemia, spleen enlargement and persistent peripheral and bone marrow monocytosis [2, 3, 10, 17, 18, 26, 27, 29, 38, 51]. This syndrome has also been described under the names of subacute myelomonocytic leukemia [38, 50, 51] or chronic erythromonocytic leukemia [12, 39]. Besides splenomegaly and monocytosis the most striking features of CMML are a significant increase in both plasmatic and urinary muramidase [13, 29, 49, 53] and the results of bone marrow culture [19, 31, 42, 50]. CMML has been reported to be associated with acquired erythrocyte alterations [10, 22, 29, 50] among which pyruvate kinase (PK) is outstanding

[1] We are grateful to Mrs. *Asumpció Pujades* for her technical assistance.

Table II. Activities (U/100 ml of PRBC) of erythrocyte enzymes and some intermediates studied in the patient, in 3 of the patient's sons and in 20 normal controls

Enzyme	Patient	Patient's sons			Normal range (1 SD)
		L.J.B.	C.J.B.	J.J.B.	
Embden-Meyerhof pathway					
Hexokinase	18	12.5	11	11	10–14
Glucosephosphate isomerase	360	502	628	608	442 594
Phosphofructokinase	*187*	*160*	*152*	*210*	*137–229*
Fructosediphosphate aldolase	114	82	94	81	71–99
Glyceraldehydephosphate dehydrogenase	3.674	2.864	3.114	3.240	2.231 3.237
Phosphoglycerate kinase	3.507	2.920	3.140	3.600	2.865–3.972
Triosephosphate isomerase	31.766	29.140	36.714	28.300	28.042 35.903
Phosphoglyceratemutase	402	502	610	395	370–542
Phosphopyruvate hydratase (enolase)	299	320	340	270	247–351
Pyruvate kinase	97	135	110	98	150–216
Diphosphoglyceratemutase	34	32	41	28	27–43
ATP (nM/ml of PRBC)	3.124	2.740	3.600	2.650	2.800–3.900
2,3-*Diphosphoglycerate (μM/g of* Hb)	*18.3*	*17.0*	*16.5*	*18.3*	*11–19*
2,3-Diphosphoglycerate/ATP ratio	2.0	2.1	1.55	2.3	2.48
H … shunt					
Glucose-6-phosphate dehydrogenase	134	164	145	147	139–175
6-Phosphogluconate dehydrogenase	144	113	95	114	85–117
Glutathione metabolism					
Glutathione-reductase (without FAD)	54	110	86	92	73–111
Glutathione-reductase (with FAD)	*66*	*123*	*98*	*112*	*96–126*
Glutathione-peroxidase	979	360	520	640	358–560
Reduced glutathione (mg/100 ml of PRBC)	79	83	66	65	58–84
Other enzymes					
Adenylate kinase	2.705	2.890	4 160	3.260	2.714–3.848
Acetylcholinesterase (U/g of Hb)	16	32	37	31	28–35

that leads to chronic hemolytic anemia [9]. In addition, the existence of reduced red cell PK activity has also been frequently noted in several hematologic disorders such as primary medullary insufficiency [5, 7, 15, 43–45], acute leukemia [1, 6, 8, 32, 34, 48, 52] and the so-called acquired hemopoietic dysplasias [1, 5, 8, 14, 22, 44]. In all these conditions, PK deficiency appeared to be acquired and without specific pathogenic significance for the hemopathies [20, 21, 43].

Reports of hereditary red cell enzyme defects associated with acquired hematologic diseases are relatively rare and little is known about the relationship between hereditary red cell enzymopathies and adult leukemia [20, 21, 43].

Our patient showed a hereditary PK deficiency with residual erythrocyte PK activ

Table I. Hematologic data from the patient

Red cell count, $\times 10^{12}$/l	3.4	
Hemoglobin, g/dl	10.9	
Packed cell volume, l/l	0.32	
Mean corpuscular volume, fl	101	
Mean corpuscular hemoglobin pg	33	
Reticulocytes, $\times 10^{9}$/l	30	
White cell count, $\times 10^{9}$/l	48.3	
Platelet count, $\times 10^{9}$/l	180.2	
Indirect bilirubin, μmol/l	4.5	(n 3–21)
Serum iron, μmol/l	12	(n 14–29)
Total iron binding capacity	42	(n 45–72)
Serum vitamin B_{12}, ng/l	850	(n 200–1,000)
Serum folate, μg/l	4	(n 3–20)
Red cell folate, μg/l	257	(n 200–704)

negative) whereas reaction to anti-I was normal. Reaction to anti-A_1 showed the presence of a double population (the patient's blood group was A_1 Rh+).

The results of erythrocyte enzyme activities and some RBC intermediates, studied in the patient and her relatives, are summarized in table II. In the propositus, PK and AChE activities were decreased to about 50% of normal values. Glucosephosphate isomerase (GPI) and glutathione reductase (GSSG R) were only slightly decreased. On the contrary, hexokinase (HK), 6-phosphogluconate dehydrogenase (6PGD) and glutathione peroxidase (GSH Px) were found to be markedly increased. Enzymatic activities studied in the patient's young and old erythrocyte populations did not differ significantly. A PK deficiency was also found in 2 of the patient's 3 sons studied, while the values of the other enzyme activities, also studied in the sons, were found to be normal.

The erythrocyte intermediates studied in the patient and her sons are also represented in table II. The patient's erythrocyte GSH content was slightly increased without instability in the presence of APH. ATP and 2,3-DPG content of the erythrocytes and therefore 2,3-DPG/ATP ratio were also found within normal limits in both the patient and her sons.

Discussion

CMML in adults is a myeloproliferative disease with the characteristics of hemopoietic dysplasia or preleukemic syndrome [3, 10, 18, 22, 50]. According to the FAB cooperative group for the classification of acute leukemias [3], the most peculiar hematologic features of CMML are the persistent peripheral and bone marrow monocytosis and the great increase in the urinary and plasmatic muramidase. The monocytic nature of the mainly atypical leukocytes found in both bone marrow and peripheral blood smear could be demonstrated by the positivity to NASDA stain which was almost completely inhibited by NaF [3]. Like other stem cell diseases, CMML is associated with multiple and simultaneous morphological and biochemical abnormalities in all blood cell lines [10, 15, 22, 36].

Whereas biochemical alterations in red blood cells are frequently described in certain hemopoietic dysplasias [5–8, 15, 22, 25, 34, 37, 44, 45], they are scarcely mentioned in CMML [10, 17, 22, 29, 51]. In the present case of CMML, deficiencies in several red cell enzymes were found. The most important feature was an erythrocyte PK deficiency in which the family study showed to be congenital. Erythrocyte PK deficiency is the most common genetic disorder of the Embden Meyerhof pathway

13 Catowsky D Galton, D A C, and Griffin, C. The significance of lysozyme estimations in acute myeloid and chronic monocytic leukaemia. Br J Haemat. 21 565–579 (1971).

14 Chapman, R G and Shaumburg, L. Glycolysis and glycolytic enzyme activity of aging red cells in man. Br J Haemat. 13 665–678 (1967).

15 Dreyfus, B., Sultan, C Rochant, H., Salmon, C., Mannoni, P Cartron, J P Boivin, P and Galand, D. Anomalies of blood group antigens and erythrocyte enzymes in two types of chronic refractory anaemia. Br J Haemat. 16 303–312 (1969).

16 Emerson, P M and Carrow D H. Differences in the two red cell populations in erythroleukemia. Lancet ii 1150 (1972).

17 Farquet, J J Schmidt, P M., Borem, G., Berriers, S et Kapanci Mlecher J Les leucémies myélomonocytaires de l'adulte. Schweiz. med. Wschr 104 135–138 (1974).

18 Geary C G Catowsky D Wiltshaw F Milner, G R Sholes, M C Van Norden, S., Wadsworth, L D Muldal, S MacIver J E., and Galton, D A G Chronic myelomonocytic leukemia. Br J Haemat. 30 289–302 (1975).

19 Gillian, R., Milner N G., Testa, C G Geary T M., Dexter S., Muldal, S., MacIver J E., and Latja, L G.: Bone marrow culture studies in refractory cytopenia and 'smouldering leukemia. Br J Haemat. 35 251–261 (1977).

20 Goebel, K M., Goebel, F D., Janzen, R., and Kaffarnik, H Haemolytic anaemia with hereditary pyruvate kinase instability developing acute leukaemia. Scand. J Haematol. 14 249–257 (1975).

21 Helmstadter V Arnold, H Blume, K G Uhl, N., and Hunstein, W Acquired pyruvate-kinase deficiency with hemolysis in preleukemia. Acta haemat. 57 339–343 (1977).

22 Hemopoietic Dysplasias (preleukemic states). Blood cells, vol. 2, No. 1–2, p. 29 (Springer Heidelberg 1976).

23 Herz, F and Kaplan, E. A microtechnic for the separation of erythrocytes in accordance with their density Am. J clin. Path. 43 181 (1965)

24 Hopkins, J and Tudhope, G. R. Glutathione peroxidase in human red cells in health and in disease. Br J Haemat. 25 563–575 (1973)

25 Kahn, A., Boivin, P Wroklaw, M. et Hakim, J Erythroleucémie avec double population érythrocytaire: Coexistence dans une même population d'un affaiblissement d'un antigène de groupe, d'un déficit majeur en adenylate-kinase et d'une augmentation de la fraction A_2 de l'hémoglobine. Nouv Revue fr Hémat. 12 609–630 (1972).

26 Kass, L. and Schnitzer, B. Monocytes, monocytosis and monocytic leukemia (Thomas, Springfield 1973).

27 Linman, J W Myelomonocytic leukemia and its preleukemic phase. J chron. Dis. 22. 713–716 (1970).

28 Martin Vega, C., Vives-Corrons, J L., Arnengol, R. y Irrigibie, D Anomalias de los antigenos de membrana eritrocitaria en las anemias refractarias. Sangre 21 664–666 (1976).

29 Mlescher P A. and Farquet, J J Chronic myelomonocytic leukemia in adults. Semin. Hemat. 11 129–139 (1974).

30 Minakami, S., Suzuki, C., Saito, T and Yoshikawa, H Studies on erythrocyte glycolysis. Determination of the glycolytic intermediates in human erythrocytes. J Biochem. 58 543–549 (1965).

31 Moore, M., Spitzer G., Williams, N., Metcalf, D., and Buckley J Agar culture studies in 127 cases of untreated acute leukemia. the prognostic value of reclassification of leukemia according to in vitro growth characteristics. Blood 44 1 17 (1974).

32 Najman, A., Leroux, J P Tempkins, H., Cartier P et André, R. Déficit en Pyruvate-Kinase érythrocytaire au cours des leucémies aiguës. Revue fr Etud. clin. biol. 14 795–796 (1969).

33 Nakao, M., Nakayama, T and Kankura, T.: A new method for separation of human blood components. Nature new Biol. 246. 94 (1973).

34 Pagnier J Labie, D Kaplan, J C., Janiea, C., Najman, A. et Leroux, J P Etude biochimique d'un cas d'érythroleucémie. Mise en évidence dans une population d'érythrocytes anormaux d'anomalies simultanées de l'hémoglobine et des enzymes de la glycolyse. Nouv Revue fr Hémat. 12. 317–332 (1972).

35 Parry R. M., Chandan, R., and Shahani, K.

ity of about 50% of the normal value. Such a decrease in PK activity is a common finding in heterozygous subjects for erythrocyte PK deficiency [9] Probably our patient is heterozygous for an abnormal PK gene which was also found in 2 of her relatives. In addition, the 2,3 DPG/ATP ratio in the patient's erythrocytes was normal whereas in subjects with hereditary PK deficiency and hemolytic manifestations (almost homozygotes or double heterozygotes) 2,3-DPG/ATP ratio increases two- or threefold [49] The other erythrocyte enzyme deficiencies observed in the patient were considered to be acquired since they failed to appear in her relatives.

The association between hereditary PK deficiency and leukemia is very rare and only the development of a hereditary PK deficiency into acute monocytic leukemia has been recently reported [20] In the present case, besides the red cell enzyme defects, other erythrocyte enzymes were found to have abnormally high activities. The greatest increase in enzyme activities were that of HK, 6PGD and GSH Px which cannot be attributed to the presence of young red cells in the patient's blood [14 46] since the reticulocyte counts were always normal or decreased. Very high values of red cell enzyme activities and especially GSH Px have been described in megaloblastic anemias [24] acute leukemia in relapse [24] and erythroleukemia [16 24] Probably high red cell enzyme activity without reticulocytosis reflects the presence of an abnormal red cell population characteristic of dyserythropoiesis [24] According to this, ultrastructural studies of the patient's bone marrow cells demonstrated important dyshemopoietic features in all blood cell precursors and especially in erythroblasts.

References

1 Arnold, H., Blume, K. G Lohr G W Boulard, G W and Najean Y.. Acquired red cell enzyme defects in hematological diseases. Clinica chim. Acta 57 187–189 (1974)

2 Bennett, J M.. Myelomonocytic leukemia. A historical review and perspective. Cancer 27 1218–1220 (1971)

3 Bennet, J M., Catowsky D., Daniel, M T Flandrin, G Galton, D A. C. Gralnick, H. R., and Sultan, C.. Proposals for the classification of the acute leukaemias French-American-British (FAB) Co-operative Group Br J Haemat. 33 451–458 (1976).

4 Beutler E. Duron, O and Kelly B M.. Improved method for determination of blood glutathione. J Lab. clin. Med. 61 882–888 (1963).

5 Boivin, P., Galand, C. et Dreyfus, B.. Activités enzymatiques érythrocytaires au cours des anémies réfractaires. Nouv Revue fr Hémat. 9 105–112 (1969)

6 Boivin, P., Galand, C. et Audollent, M.. Erythroenzymopathies acquises. Anomalies quantitatives observées dans 100 cas d'hémopathies diverses. Path. Biol., Paris 18. 175–187 (1970).

7 Boivin, P Hakim J et Kahn, A.. Anomalies biochimiques des cellules sanguines au cours des anémies réfractaires. Annls Méd. interne 125 627–632 (1974)

8 Boivin, P., Galand, C. Hakim, J and Kahn, A.. Acquired erythroenzymopathies in blood disorders: study of 200 cases. Br J Haemat. 31 531–543 (1975).

9 Boivin, P., Gadjos, A. et Lestradet, H.. Enzymopathies. Fascicule 1er (Masson, Paris 1971).

10 Briere, J., Dresch, C., Briere, F et Faille, A.. Leucemies myelomonocytaires subaigues ou chroniques de l'adulte. Actualités Hématologiques, 11ème série pp. 53–80 (Masson, Paris 1977).

11 Browdson, C. and Watts, D C. The modification of cholinesterase activity by 5,5'-dithiobis-(2 nitrobenzoic acid) included in the coupled spectrophotometric assay Biochem. J 131 369–374 (1973)

12 Broun, G O Chronic erythromonocytic leukemia. Am. J Med. 47 785–796 (1969).

13 Catovsky D Galton, D A. C., and Griffin, C. The significance of lysozyme estimations in acute myeloid and chronic monocytic leukaemia. Br J Haemat. *21* 565–579 (1971)

14 Chapman, R. G and Shaumburg, L. Glycolysis and glycolytic enzyme activity of aging red cells in man. Br J Haemat. *13* 665–678 (1967).

15 Dreyfus, B Sultan, C., Rochant, H Salmon, C., Mannoni, P Cartron, J P Boivin, P and Galand, D Anomalies of blood group antigens and erythrocyte enzymes in two types of chronic refractory anaemia. Br J Haemat. *16* 303–312 (1969).

16 Emerson, P M. and Carrow D. H.. Differences in the two red cell populations in erythroleukemia. Lancet *ii*. 1150 (1972).

17 Farquet, J J Schmidt, P M., Boreux, G Berriers, S. et Kapanci Mischer J Les leucémies myelomonocytaires *de l'adulte*. Schweiz. med. Wschr *104* 135–138 (1974)

18 Geary C. G Catovsky D., Wiltshaw P Milner G. R. Sholes, M C., Van Norden, S., Wadsworth, L. D Muldal, S., MacIver J E., and Galton, D. A. G Chronic myelomonocytic leukemia. Br J Haemat. *30* 289–302 (1975).

19 Gillian, R., Milner N G., Testa, C. G., Geary T M., Dexter S., Muldal, S., MacIver J E., *and Lajtha, L. G.. Bone marrow* culture studies in refractory cytopenia and 'smouldering leukemia Br J Haemat. *35* 251–261 (1977).

20 Goebel, K. M., Goebel, F D Janzen, R., and Kaffarnik, H.. Haemolytic anaemia with hereditary pyruvate kinase instability developing acute leukemia Scand. J Haematol. *14* 249–257 (1975).

21 Helmstädter V Arnold, H., Blume, K. G Uhl, N and Hamstern, W Acquired pyruvate-kinase deficiency with hemolysis in preleukemia. Acta haemat. *57* 339–343 (1977).

22 Hemopoetic Dysplasias (preleukemic states) Blood cells, vol. 2, No. 1–2, p. 29 (Springer Heidelberg 1976)

23 Herz, F and Kaplan, E. A microtechnic for the separation of erythrocytes in accordance with their density Am. J clin. Path. *43* 181 (1965).

24 Hopkins, J and Tudhope, G R Glutathione-peroxidase in human red cells in health and in disease. Br. J Haemat. *25* 563–575 (1973).

25 Kahn, A., Boivin, P Wroklans, M. et Hakim, J.: Erythroleucémie avec double population érythrocytaire: Coexistence dans une même population d'un affaiblissement d'un antigène de groupe, d'un déficit majeur en adenylate-kinase et d'une augmentation de la fraction A_2 de l'hémoglobine. Nouv Revue fr Hémat. *12*. 609–630 (1972).

26 Kass, L. and Schnitzer B.. Monocytes, monocytosis and monocytic leukemia (Thomas, Springfield 1973).

27 Linman, J W Myelomonocytic leukemia and its preleukemic phase. J chron. Dis. 22. 713–716 (1970).

28 Martin Vega, C., Vives-Corrons, J L., Armengol, R. y Irrigible, D Anomalías de los antígenos de membrana eritrocitaria en las anemias refractarias. Sangre *21* 664–666 (1976).

29 Miescher P A. and Farquet, J J Chronic myelomonocytic leukemia in adults. Semin. Hemat. *11* 129–139 (1974).

30 Minakami, S., Suzuki, C., Saito, T and Yoshikawa, H.. Studies on erythrocyte glycolysis. Determination of the glycolytic intermediates in human erythrocytes. J Biochem. *58* 543–549 (1965).

31 Moore, M., Spitzer G Williams, N., Metcalf, D and Buckley J Agar culture studies in 127 cases of untreated acute leukemia: the prognostic value of reclassification of leukemia according to *in vitro* growth characteristics. Blood *44* 1–17 (1974).

32 Najman, A., Leroux, J P Tempkine, H., Cartier P et André, R.. Déficit en Pyruvate-Kinase érythrocytaire au cours des leucémies aiguës. Revue fr. Etud. clin. biol. *14* 795–796 (1969).

33 *Nakao, M., Nakayama, T and Kankura, T A new method for separation of human blood* components. Nature new Biol. *246*. 94 (1973).

34 Paglier J Labie, D., Kaplan, J C., Junien, C., N Jman, A. et Leroux, J P Etude biochimique d'un cas d'érythroleucémie. Mise en évidence dans une population d'érythrocytes anormaux d'anomalies simultanées de l'hémoglobine et des enzymes de la glycolyse. Nouv Revue fr Hémat. *12*. 317–332 (1972)

35 Parry R. M., Chandan, R., and Shahani, K.

ity of about 50% of the normal value Such a decrease in PK activity is a common finding in heterozygous subjects for erythrocyte PK deficiency [9] Probably our patient is heterozygous for an abnormal PK gene which was also found in 2 of her relatives. In addition the 2,3-DPG/ATP ratio in the patient's erythrocytes was normal, whereas in subjects with hereditary PK deficiency and hemolytic manifestations (almost homozygotes or double heterozygotes) 2,3-DPG/ATP ratio increases two- or threefold [49] The other erythrocyte enzyme deficiencies observed in the patient were considered to be acquired since they failed to appear in her relatives.

The association between hereditary PK deficiency and leukemia is very rare and only the development of a hereditary PK deficiency into acute monocytic leukemia has been recently reported [20] In the present case, besides the red cell enzyme defects, other erythrocyte enzymes were found to have abnormally high activities. The greatest increase in enzyme activities were that of HK, 6PGD and GSH Px which cannot be attributed to the presence of young red cells in the patient's blood [14 46] since the reticulocyte counts were always normal or decreased. Very high values of red cell enzyme activities and especially GSH Px have been described in megaloblastic anemias [24] acute leukemia in relapse [24] and erythroleukemia [16 24] Probably high red cell enzyme activity without reticulocytosis reflects the presence of an abnormal red cell population characteristic of dyserythropoiesis [24] According to this, ultrastructural studies of the patient's bone marrow cells demonstrated important dyshemopoietic features in all blood cell precursors and especially in erythroblasts.

References

1 Arnold, H., Blume K. G., Lohr G. W., Boulard, G W and Najean Y., Acquired red cell enzyme defects in hematological diseases. Clinica chim. Acta 57 187–189 (1974).

2 Bennett, J M., Myelomonocytic leukemia. A historical review and perspective. Cancer 27 1218–1220 (1971)

3 Bennet, J M., Catowsky D Daniel, M T Flandrin, G Galton, D A. G., Gralnick, H. R., and Sultan, C., Proposals for the classification of the acute leukaemias French-American-British (FAB) Co-operative Group. Br J Haemat. 33 451–458 (1976)

4 Beutler E. Duron, O and Kelly B. M., Improved method for determination of blood glutathione. J Lab clin. Med. 61 882–888 (1963).

5 Boivin, P Galand, C. et Dreyfus, B., Activités enzymatiques érythrocytaires au cours des anémies réfractaires. Nouv Revue fr Hémat. 9 105–112 (1969).

6 Boivin, P Galand, C. et Audollent, M., Erythroenzymopathies acquises. Anomalies quantitatives observées dans 100 cas d'hémopathies diverses. Path. Biol., Paris 18 175–187 (1970).

7 Boivin, P., Hakim J et Kahn, A. Anomalies biochimiques des cellules sanguines au cours des anémies réfractaires Annls Méd. interne 125 627–632 (1974)

8 Boivin, P., Galand, C., Hakim, J and Kahn, A., Acquired erythroenzymopathies in blood disorders. study of 200 cases. Br J Haemat. 31 531–543 (1975).

9 Boivin, P Gadjos, A. et Lestradet, H Enzymopathies. Fascicule 1er (Masson, Paris 1971).

10 Briere, J., Dresch, C., Briere, F et Faille, A. Leucémies myelomonocytaires subaigues ou chroniques de l'adulte. Actualités Hématologiques, 11ème série, pp 53–80 (Masson, Paris 1977)

11 Browdson, C. and Watts, D C The modification of cholinesterase activity by 5,5'-dithiobis-(2-nitrobenzoic acid) included in the coupled spectrophotometric assay Biochem. J 131 369–374 (1973).

12 Broun, G O., Chronic erythromonocytic leukemia. Am. J Med. 47 785–796 (1969)

Short Communication

Acta haemat. 61 175–176 (1979)

Pernicious Anaemia in an Eleven Year Old Male

Michael A. Farrell, Con Murphy and Sheamus Dundon

Department of Renal and Metabolic Medicine, Our Lady's Hospital for Sick Children, Crumlin, Dublin

Key Words. Pernicious anaemia · Achlorhydria · Mastoid pain

Abstract. A case of classical addisonian pernicious anaemia is described in an 11 year old male.

Pernicious anaemia (PA) in childhood is rare. Two groups, based on gastric mucosa histology and acid secretion occur. In the first there is a genetically determined failure of intrinsic factor (IF) secretion combined with normal acid output and unremarkable gastric mucosa. 25 cases have been reported to date [4, 5].

The second group is similar in all respects to classical addisonian PA seen in adults, i.e. gastric mucosal atrophy, achlorhydria and failure of IF secretion occur. In addition, parietal cell and IF antibodies are occasionally present. 10 cases have been reported in the world literature [4, 5] but in only one [4] of these were full antibody studies completed. We wish to report an additional fully documented case of adult type PA of juvenile onset.

Case History

An 11 year-old boy was admitted because of persistent left mastoid pain of increasing severity extending over a period of 4 weeks. In addition, he complained of breathlessness on exertion. His mother stated that he had become pale, listless, and had lost his appetite.

The patient was the second eldest of 4 children. Both parents were 37 years old. There was no history of consanguinity. 5 years previously the patient had been hospitalized because of bronchopneumonia and was noted to have a hypochromic anaemia, (Hgb 9.0 g/dl). This responded to oral iron therapy.

On examination, his height and weight were at the 50th percentile. Marked pallor of the skin and mucous membranes was noted. He was afebrile. The left mastoid area was tender. Lymphadenopathy and hepatosplenomegaly were not present. A soft apical systolic bruit was audible. Clinically it was felt that the patient may have had a mastoiditis.

During the first week in hospital, he developed recurrent episodes of central abdominal pain associated with vomiting, usually lasting 1–2 h. No abnormal physical signs were noted on any of these occasions.

A left mastoid X-ray was normal. The Hgb was 6.9 g/dl, RCC 1.6×10^{12}/l, MCV 119 fl, Hct 19%, MCH 37 pg, MCHC 36 g/dl, white cell count 3.3×10^{9}/l, reticulocytes less than 1%, and platelets 100×10^{9}/l. Macrocytes were present on the

M A rapid and sensitive assay of muramidase. Proc. Soc. exp. Biol. Med. *129* 384–386 (1965)
36 Saarni, M L and Linman, J W. Myelomonocytic leukemia disorderly proliferation of all marrow cells. Cancer 27 1221–1230 (1971)
37 Selroos, O and Vuopio P. Glucose-6-phosphate dehydrogenase deficiency and myelofibrosis. Scand. J Haematol. *11* 106–111 (1973)
38 Sexauer J Kass, L, and Schnitzer B. Subacute myelomonocytic leukemia. Clinical, morphologic and ultrastructural studies of 10 cases. Am. J Med. 57 853–861 (1974)
39 Shaw M T Bottomley S. S. Bottomley R. H and Hussein, K. K. The relationship of erythromonocytic leukemia to other myeloproliferative disorders. Am. J Med. *55* 542–548 (1973).
40 Sigma Technical Bulletin. No. 35 UV 12 (1971)
41 Singer K., Chernoff, A. L., and Singer L. Studies of abnormal hemoglobins. I Their demonstration in sickle cell anemia and other hematologic disorders by means of alkali denaturation. Blood 6. 413–428 (1951).
4 Sultan, C., Marquet, M. et Joffroy Y. Etude de certaines dysmyélopoïèses acquises idiopathiques et secondaires par culture de moelle *in vitro* Annls Méd. interne. *125* 599–602 (1974)
43 Tanaka, K. B Valentine, W N., and Miwa, S Pyruvate kinase deficiency and hereditary non-spherocytic hemolytic anemia. Blood *19* 267–295 (1962).
44 Valentine, W N., Konrad, P N., and Paglia D E. Dyserythropoiesis refractory anemia and 'preleukemia metabolic features of the erythrocytes. Blood *41* 857–875 (1973)
45 Vives-Corrons, J L. Hernandez Nieto L. Montserrat, E. Pujades, A. y Rozman, C. Actividad de los enzimas eritrocitarias en el curso de las anemias refractarias. Sangre *21* 647–655 (1976)
46 Vives-Corrons, J L. Pujades, M. A. y Rozman, C. Enzimas eritrocitarias. I. Estudio comparativo entre los valores normales de actividad en adultos sanos, en adultos con reticulocitosis y en Sangre de cordón umbilical. Med. Clin. *68* 52–56 (1977).
47 Vives-Corrons, J L. Rozman, C. Kahn, A., Carrera, A., and Trigïner S. Glucosephosphate isomerase deficiency with hereditary hemolytic anemia in a Spanish family Clinical and familial study Hum. Genet. *29* 291–297 (1975)
48 Vives-Corrons, J L., Carreras, M. D Brugues, R. M., Florensa, L. Pujades, M. A. y Rozman, C. Actividad de los enzimas eritrocitarias en la leucemia aguda. Sangre, *23* 425–437 (1978)
49 Vives-Corrons, J L. Marie, J Pujades, M A., and Kahn, A. Hereditary erythrocyte pyruvate kinase deficiency search for correlations between molecular studies and clinical manifestations (in preparation, 1978)
50 Zittoun, R. Annotation. subacute and chronic myelomonocytic leukaemia, a distinct haematological entity Br J Haemat. *32* 1–7 (1976).
51 Zittoun, R., Benadou, A. Bilski-Pasquier C. et Bousser S. Les leucémies myélomonocytaires subaiguës. Etude de 27 cas et revue de la Littérature. Sem. Hôp Paris *48* 1943–1956 (1972).
52 Zittoun, R., Bernadou, A., Leclerc, M Povillart, P Soria, J., Cosson, G Vagner D Bilsky Pasquier G et Bousser J Recherche de différentes anomalies érythrocytaires dans les leucemies. Revue eur Etud. clin. biol. *15*. 215–219 (1970).
53 Zittoun, J., Jarret, J., Zittoun, R. et Sultan, C. Transcobalamines et lysozyme dans les leucémies et les syndromes myéloprolifératifs. Nouv Revue fr Hémat. *14* 324 (1974).

Received. February 1 1978
Accepted. July 1 1978

Dr J L. Vives-Corrons, Postgraduate School of Hematology Farreras-Valenti' Hospital Clinico y Provincial University of Barcelona, c/ Casanova, 143 Barcelona (Spain)

Original Papers

Acta haemat. 61 177 183 (1979)

Use of Specific Antisera against Leukemia-Associated Antigens in the Diagnosis of Childhood Acute Lymphoblastic Leukemia[1]

Bruno Netzel Hans Rodt Eckhard Thiel Rainer J Haas and Stefan Thierfelder

Department of Hematology and Oncology Children's Hospital, University of Munich, and Institute of Hematology of the 'Gesellschaft fur Strahlen- und Umweltforschung Munich

Key Words. Acute lymphoblastic leukemia in childhood Cell membrane markers

Abstract. A series of 66 children with acute lymphoblastic leukemia (ALL) at diagnosis were investigated (simultaneously) for various surface markers. For this purpose the reaction of specific antisera against ALL antigens and T cell antigens was analysed in every case by several test systems, namely immunofluorescence, microcytotoxicity and complement fixation. A clearly defined classification in 6 subgroups of ALL emerged. The clinical data at presentation and possible correlations with the immunological subgroups were demonstrated.

Recent chemotherapeutic treatment programs have resulted in a marked improvement of prognosis in childhood acute lymphoblastic leukemia [6] However children with ALL unresponsive to chemotherapeutic treatment may represent a definite subgroup of the disease and therefore may require different therapeutic strategies.

Recent studies have shown that ALL can be subdivided into subsets by membrane phenotyping [1 8, 20 26] In this report we present details on phenotyping of 66 children using different surface markers, including ALL antigens, as compared with clinical data of the disease at diagnosis.

[1] Supported by the 'Deutsche Krebshilfe' Bonn.

Patients, Methods and Materials

Membrane phenotyping was done in cell samples of 66 children with ALL. Our therapeutic program is described in detail elsewhere [9].

Remission is induced by vincristine, prednison, asparaginase and adriamycin. High-dosage methotrexate therapy followed by cyclophosphamide cytosin-arabinoside, vincristine and prednison, is used to effect early consolidation. Central nervous system prophylaxis consists in 2,400-rad whole brain irradiation, using ^{60}Co unit, associated with intrathecal administration of methotrexate [5]. Treatment in remission involves 6-mercaptopurine daily and methotrexate weekly Therapy is discontinued after 30 months [16].

Cell Preparations

Normal lymphocytes and ALL cells were separated from peripheral blood and bone marrow by

peripheral blood film. Bone marrow examination revealed marked megaloblastic haemopoiesis.

Vitamin B_{12} was assayed, at 50 ng/l (normal 150–1 000 ng/l) and the serum folate was 8 μg/l (normal 3–20 μg/l). In phase I of the standard Schilling test, 2% of the test dose was recovered in the urine. In phase II 17% was recovered (normal 15%). Pentagastrin fast achlorhydria was present. IF was not detected in the gastric juice. The serum contained gastric parietal cell and IF blocking antibodies. Serum LDH was moderately elevated.

The following investigations were normal: serum iron and transferrin, urea, sodium, potassium, chloride, thyroxine, cortisol (morning and evening), insulin, glucose, phosphate, magnesium, uric acid, immunoglobulins, barium meal and follow through to the terminal ileum, jejunal biopsy, stools for ova and parasites, and stools for occult blood.

5 days after the Schilling test, the reticulocyte count was 30%. Hydroxycobalamin therapy was commenced, and 4 weeks later the Hgb was 15 g/dl and erythropoiesis was normoblastic. The mastoid and abdominal pains disappeared spontaneously. 2 years later the patient is well and receives hydroxycobalamin bimonthly. The other members of the family were clinically well. None were anaemic. An auto-antibody screen showed that the father was anti-nuclear factor positive and the mother had positive thyroid microsomal and smooth muscle antibodies.

Discussion

Episodes of abdominal pain in PA resembling a tabetic crisis are known to occur [1]. Mastoid pain has not, to our knowledge, been reported as a presenting feature of PA. There is much evidence to suggest that adult PA occurs secondary to an auto-immune gastritis, partly genetically determined and partly environmental [3].

It is possible that this patient has a genetically determined enhanced ability to form auto-antibodies. Presumably he first developed the gastric parietal cell antibody when he had the iron deficiency anaemia in 1970 and that the gastric lesion had been developing since then until it had become sufficiently advanced to be responsible for his megaloblastic anaemia.

References

1 Bailey, H.: Abdominal crisis of pernicious anaemia. Br. med. J. 2: 554–557 (1926).
2 Chisholm, M.: in Callender: Clinics in haematology, vol. 2, p. 312 (Saunders, London 1973).
3 Hoffbrand, A. V.: in Hoffbrand and Lewis, Haematology tutorials in postgraduate medicine, vol. 2, p. 63 (Heinemann, London 1975).
4 Nasu, T. et al.: Pernicious anaemia in childhood. Jap. J. clin. Haematol. 18: 59–65 (1977).
5 Spector, J.: Juvenile achlorhydric pernicious anaemia with IgA deficiency. J. Am. med. Ass. 228: 334–336 (1974).

Recieved: July 10, 1978
Accepted: August 18, 1978

Michael A. Farrell, MRCPI, Department of Pathology, St. Vincent's Hospital, Elm Park, Dublin 4 (Ireland)

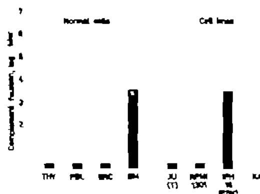


Fig. 1. Reactivity of anti-cALL absorbed only with normal lymphocytes and not with lymphoblastoid cell line against various normal cells and cell lines. THY = thymocytes; PBL = peripheral blood lymphocytes; GRC = granulocytes; BM = bone marrow cells. JU = T cell line; RPMI 1301 = B cell line; IFH 16 = lymphoblastoid cell line; KA = cALL cell line.

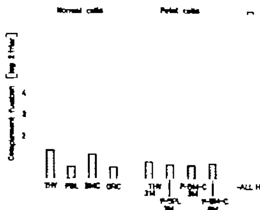


Fig. 2. Reactivity of anti-cALL absorbed with the lymphoblastoid cell line IFH 16 against various normal and cALL cells. THY = thymocytes; PBL = peripheral blood lymphocytes; BMC = bone marrow cells, GRC = granulocytes; F-THY, F-SPL, F-BM C = fetal thymus, spleen, bone marrow cells (3 and 6 months). ALL HI = bone marrow cells of a patient with cALL.

Results

Specificity of Anti-cALL

Evidence was found that the highly absorbed anti-cALL is a specific antiserum only against those antigens which are associated with leukemia blast cells lacking B or T cell markers. Figure 1 shows the results of the complement fixation test where anti-cALL absorbed only with normal lymphocytes and *not* with lymphoblastoid cell lines was used. There was no reaction with normal thymocytes, peripheral blood lymphocytes, granulocytes, the T (JU), or the B-cell-line RPMI 1301. However a marked cross-reaction against cell suspensions of normal bone marrow and the lymphoblastoid cell line IFH 16 was found.

If however the anti-cALL is in addition absorbed with the B lymphoblastoid cell line IFH 16, it can be clearly demonstrated that the additional absorption removes the reactivity against normal bone marrow cells (fig. 2). There also were no cross-reactions against fetal thymus, spleen and bone marrow cells. In contrast, the high activity

Table I. Toxicity of crude and differently absorbed anti-cALL globulin on stem cells committed to myeloid differentiation (CFU-C/2 10^5 nucl. marrow cells mean ± SEM of triplicate counts)

	CFU-C
Not absorbed	0
Absorbed without the lymphoblastoid cell line	27.3 ± 1.5
Absorbed with the lymphoblastoid cell line	69.5 ± 1.7
Normal rabbit globulin	72.1 ± [illegible].7

Fresh rabbit serum diluted to 1:4 served as the source of complement. All preparations were adjusted to protein content of 10 mg/ml.

density gradient centrifugation on a Ficoll Isopaque gradient. Normal granulocytes were prepared by removing erythrocytes through dextran sedimentation, followed by removal of lymphocytes on a Ficoll Isopaque gradient. ALL blasts, used for the production of antisera, were obtained from untreated patients at the University Children s Hospital Munich

The JU cell line, obtained from an E rosette forming T variant ALL (T ALL) [23] and the KA cell line, obtained from a common ALL (cALL) [2_] were kindly provided by Dr *Schneider* University Children s Hospital Erlangen (FRG) The IFH cell line was obtained by infection of normal lymphocytes with EBV-containing supernatant of the B95-8-virus-producing line at the Institute for hematology in Munich.

Antisera

Anti human T cell globulin (ATCG) was prepared from rabbit anti human thymocyte globulin by extensive absorption with liver kidney homogenates, chronic lymphatic leukemia cells (of the B cell type) and B lymphoblastoid cell lines. The absorption and subsequent purification procedures have been described elsewhere [18]. Detailed investigations of the specificity and the reaction pattern of ATCG have been published previously [18].

Anti-cALL sera were raised in rabbits with ALL cells negative for surface immunoglobulin, T antigen and E rosette formation. 10^8 cells incubated with crude rabbit anti-thymocyte globulin at a final dilution of 1 30 to cover nonleukemic determinants were injected subcutaneously [7] The rabbits were boosted intravenously on days 14 15 16 and 23 and were bled 7 days later The antiserum was inactivated at 56 C for 1 h. The absorption procedure was carried out once with thoroughly washed liver kidney homogenate, three times with normal peripheral blood lymphocytes obtained by leukapheresis, using a Haemonetics model 30 blood cell processor and three times with cells from patients with chronic lymphatic leukemia, in a ratio of sediment to serum of 1 4 For reasons to be described later the serum was additionally absorbed with the lymphoblastoid cell lines IFH 16. After the last absorption, the serum was ultracentrifugated and the pure IgG fraction isolated by ammonium sulfate precipitation and DEAE cellulose ion-exchange chromatography All globulin preparations were adjusted to a concentration of 0 01 mg/l [19]

Immunofluorescence

Binding of anti-cALL was studied by indirect immunofluorescence using anti-cALL (protein concentration 0.01 mg/l) at a final dilution of 1 40 and fluoresceinated goat-anti-rabbit IgG (Behring AG) at a dilution of 1.20 as described [26, 27]. Staining was performed at 4 C and sodium azide was added in a concentration of 2×10^{-2} *M* The cells were examined with a Leitz fluorescence microscope and at least 400 cells were investigated for each sample.

Cytotoxicity Test

The microcytotoxity test was used with rabbit complement and eosin for dye exclusion [13]. The cells were suspended in RPMI 1640 and 10% heat inactivated fetal calf serum and finally adjusted to a concentration of 2 × 10 cells/10^6/liter Selected normal rabbit serum diluted to 1 4 served as a source of complement. At least 400 cells were counted in an inverted phase-contrast microscope.

Complement Fixation Test

Complement fixation was performed according to a micromethod [17]. It was adapted to human lymphocytes as antigens. Veronal-buffered saline (VBS) was used for all dilutions (100 µl of each antibody dilution) the mixture was then allowed to incubate for 30 min. Next, 100 µl of sheep erythrocytes (1 4 × 10 /liter) sensitized with anti-sheep erythrocyte serum, were added. After 30 min, the reaction mixture was diluted with 0.9 ml of VBS, sedimented with an Eppendorf centrifuge and examined for hemolysis at 412 mm in a Zeiss PM 6 spectrophotometer equipped for automatic reading. The antibody dilution with 50% lysis of sheep erythrocytes was defined as the titer Sensitization of erythrocytes was performed as described [17]. GPC was adjusted to a dilution (about 1 100) that produced 90% hemolysis when cell suspension and antibody dilution were replaced by VBS. Rosette formation and surface immunoglobulin (SIg) were investigated using standard procedures [27]

described phenotyping procedure [19 26] By membrane phenotyping, six groups of childhood ALL could be differentiated (table II).

The clinical characteristics of the patients are listed in table III. The T ALL (E and E) group was older had anterior mediastinal masses and elevated white blood cell (WBC) counts, but 20*/ of children of the cALL group also had a WBC count at diagnosis of > 25 000 × 10*/liter There are indications of a male predominance in the T ALL group but because of the small number of cases this is not yet statistically significant.

Discussion

In the presented study a consecutive series of 66 children was tested for the described surface markers. Hereby a classification in six recently defined ALL subgroups [19 26] was accomplished. As demonstrated, 38* of the patients were positive only with anti-cALL globulin and negative with ATCG Interestingly the cells of 32* of the patients reacted with anti-cALL and ATCG 25*/ of the patients showed a reaction with ATCG alone 9* of which did not express E receptors. Most of the children of the T ALL (E) group were boys, had an anterior mediastinal mass and elevated white blood counts (25 × 10*/ l) as reported by other groups [25 28]

Recently available data show that patients whose lymphoblasts had T cell antigens and or E receptors relapsed earlier whereas about 80* of patients with lymphoblasts lacking T markers survived disease-free for at least 30 months observation [4 10 1 25 27 28] Therefore, the analysis of membrane markers at diagnosis seems to permit the recognition of ALL subgroups with different prognosis. New chemotherapeutic treatment programs should include the analysis of membrane markers at diagnosis. Thus, new information would be available if the various chemotherapeutic schedules were to be suboptimal for certain variants of ALL, or if, on the other hand, intensive chemotherapy were to be unnecessary for certain immunological subsets of the disease.

Extensive investigations published previously [18] showed that ATCG reacts specifically with thymocytes and T lymphocytes. The percentage of our T-ALL group (E and E) correlates with the data reported from obter centres [1 2, 8, 11] Recently it has been shown that the expression of the E rosette marker is not invariably connected with the T cell antigen on the cell surface of leukemic blasts [2, 11 12, 20, 27] The fact that T cell antigen can be expressed in the absence of E rosette formation can be shown in cells of the MOLT-4 cell line which formed rosettes in only 22*/ of the cells but showed T antigen expression in all of the cells [18] It has been suggested that the expression of one or both T cell markers may correlate with different maturation stages of the T cell pathway where the T antigen positive, E negative cell precedes the T cell with both markers [26, 27]

About 70*/ of the investigated ALL cases reacted with anti-cALL. Similar results were found with other groups, applying comparable antisera [1 8]

In our material some of the leukemic blasts showed a strong reaction with anti-cALL and a weaker one with anti-T

However blast cells expressing cALL antigen never showed E rosette formation.

against c ALL was not affected No reactiv ity could be detected against hemopoietic stem cells committed to myeloid differentia tion as shown in table I

Cytotoxic Effect of Anti-cALL Globulin

The absorbed anti-cALL globulin was investigated for cytotoxicity against leukemic blast cells for possible therapeutic use in ALL patients. Figure 3 shows the cytotoxic effect of the antiserum against cALL bone marrow cells and leukemic cells taken from a patient with hematological relapse. It can be noted that there is complete lysis of leukemic cells by the antiserum. It can also be shown that T ALL and normal bone marrow cells are not affected.

Table II. Membrane phenotypes of our ALL patients (n = 66)

Groups	Patients n	Patients %	Memprane phenotypes
I	26	38	cALL (anti-cALL)
II	21	3	cALL with T-cell marker in addition (anti-cALL anti-T)
III	9	14	T ALL with E-rosette marker (anti-cALL anti-T+ E)
IV	6	9	T ALL without R-rosette marker (anti-cALL anti T E)
V	4	6	'undifferentiated ALL (anti-cALL anti-T- E-)
VI	1	1	B-ALL (SmIg+)

Membrane Phenotypes of Lymphoblastoid Leukemia

66 children with ALL at the time of diagnosis were investigated applying the

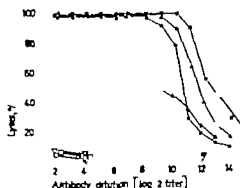


Fig. 3. Cytotoxicity of anti-cALL on cALL bone marrow cells at diagnosis (■ ▲ ●) and during hematological relapse (48% blasts; △) on T ALL cells (O), and on normal bone marrow (□) and peripheral blood cells (▽) Protein concentration. 10 mg/ml

Table III. Clinical data of 61 children with ALL characterized by immunological markers

	cALL	cALL/T	T/E	T/E
Number of patients	25	21	6	9
Male	16	9	2	8
Female	9	12	4	1
Age, years < 2 > 9	11	6	5	6
Anterior mediastinal mass	0	0	2	8
WBC × 10⁹/l	29 400	23 000	81,9	134,300
Range × 10⁹/l	2,400–155 000	1,900–87,200	9,200–150 000	30,500–400,000
Number with > 25,000 × 10⁹/l	5 (20%)	4 (19%)	4 (67%)	9 (100%)

4 patients were common ALL T ALL and B-ALL 1 patient was B-ALL. For details see text. All cases were tested with anti-cALL globulin and ATCG in the complement fixation test and by immunofluorescence.

described phenotyping procedure [19 26] By membrane phenotyping, six groups of childhood ALL could be differentiated (table II)

The clinical characteristics of the patients are listed in table III. The T-ALL (E and E) group was older had anterior mediastinal masses and elevated white blood cell (WBC) counts, but 20% of children of the cALL group also had a WBC count at diagnosis of > 25,000 × 10⁹/liter There are indications of a male predominance in the T ALL group, but because of the small number of cases this is not yet statistically significant.

Discussion

In the presented study a consecutive series of 66 children was tested for the described surface markers. Hereby a classification in six recently defined ALL subgroups [19 26] was accomplished As demonstrated, 38% of the patients were positive only with anti-cALL globulin and negative with ATCG Interestingly the cells of 32% of the patients reacted with anti-cALL and ATCG 25% of the patients showed a reaction with ATCG alone, 9% of which did not express E receptors. Most of the children of the T ALL (E) group were boys, had an anterior mediastinal mass and elevated white blood counts (25 × 10³/µl) as reported by other groups [25 28]

Recently available data show that patients whose lymphoblasts had T cell antigens and/or E receptors relapsed earlier whereas about 80% of patients with lymphoblasts lacking T markers survived disease free for at least 30 months observation [4 10, 21 25 27 28] Therefore the analysis of membrane markers at diagnosis seems to permit the recognition of ALL subgroups with different prognosis. New chemotherapeutic treatment programs should include the analysis of membrane markers at diagnosis. Thus, new information would be available if the various chemotherapeutic schedules were to be suboptimal for certain variants of ALL, or if, on the other hand, intensive chemotherapy were to be unnecessary for certain immunological subsets of the disease.

Extensive investigations published previously [18] showed that ATCG reacts specifically with thymocytes and T lymphocytes. The percentage of our T ALL group (E and E) correlates with the data reported from ohter centres [1 2, 8, 11] Recently it has been shown that the expression of the E rosette marker is not invariably connected with the T cell antigen on the cell surface of leukemic blasts [2, 11 12, 20, 27] The fact that T cell antigen can be expressed in the absence of E rosette formation can be shown in cells of the MOLT-4 cell line which formed rosettes in only 22% of the cells but showed T antigen expression in all of the cells [18] It has been suggested that the expression of one or both T cell markers may correlate with different maturation stages of the T cell pathway where the T antigen positive, E negative cell precedes the T cell with both markers [26, 27]

About 70% of the investigated ALL cases reacted with anti-cALL. Similar results were found with other groups, applying comparable antisera [1 8]

In our material some of the leukemic blasts showed a strong reaction with anti cALL and a weaker one with anti-T

However blast cells expressing cALL antigen never showed E rosette formation.

The T antigen expression of blast cells reacting with anti-cALL is suggestive of a differentiation stage ranging between cALL and T ALL, as recently supposed in a putative differentiation scheme [27].

The different reactivity pattern of anti-cALL absorbed without lymphoblastoid cell lines is characterized by a crossreaction with bone marrow cells and LCL. In contrast, the antiserum preparation which was additionally absorbed with LCL lacks this reactivity. This was clearly demonstrated in functional test systems like CFU-C and diffusion chambers [14] as well as in autoradiographic studies [19].

References

1 Borella, L., Sen, L., Dow, L. W., and Casper, J. T.: Cell differentiation antigens versus tumor-related antigens in childhood acute lymphoblastic leukemia (ALL). Clinical significance of leukemia markers; in Thierfelder and Thiel, Immunological diagnosis of leukemias and lymphomas (Springer, Berlin 1977).

2 Brouet, J. C., Valensi, F., Daniel, M. T., Flandrin, G., Preud'Homme, J. L., and Seligmann, M.: Immunological classification of acute lymphoblastic leucemias: evaluation of its clinical significance in a hundred patients. Brit. J. Haemat. 33: 319–322 (1976).

3 Brown, G. and Greaves, M. F.: Expression of human T and B lymphocyte cell surface markers on leukemic cells. Lancet ii: 753–755 (1974).

4 Coccia, P. F., Kersey, M. D., Kazamiera, J., Pecralska, G., Krivit, W., and Nesbit, M. E.: Prognostic significance of surface marker analysis in childhood non-Hodgkin's lymphoproliferative malignancies. Am. J. Hematol. 1: 405–411 (1976).

5 Dritshilo, A., Cassady, J. R., and Camitta, B.: The role of irradiation in central nervous system treatment and prophylaxis for acute lymphoblastic leukemia. Cancer 37: 2729–2735 (1976).

6 Furmann, L., Camitta, B. M., and Jaffe, N.: Development of an effective treatment program for childhood leukemia: a preliminary report. Med. pediat. Oncol. 2: 157–166 (1976).

7 Greaves, M. F., Brown, G., Rapson, N. T., and Lister, T. A.: Antisera to acute lymphoblastic leukemia cells. Clin. Immunol. Immunopathol. 4: 67–84 (1975).

8 Greaves, M. F., Janossy, G., and Roberts, M.: Membrane phenotyping: diagnosis, monitoring and classification of acute 'lymphoid' leukemias; in Thierfelder, Rodt and Thiel, Immunological diagnosis of leukemias and lymphomas (Springer, New York 1977).

9 Haas, R. J., Netzel, B., Janka, G. E., Rodt, H., Thiel, E. und Thierfelder, S.: Diagnostischer Einsatz spezifischer Antiseren bei der akuten lymphatischen Leukämie im Kindesalter. Klin. Pädiat. 19: 446–45 (1978).

10 Hardisty, R. M., Rapson, N. T., Greaves, M. F., and Chessells, J. M.: Acute lymphoblastic leukemia: correlation of cell membrane markers with clinical features and with prognoses. Abstract, ESPHO 6th meeting, Zürich 1977.

11 Kersey, J., Nesbit, M., Hallgren, H., Sabad, A., Yunis, E., and Gajl-Peczalska, K.: Evidence for the origin of certain childhood acute lymphoblastic leukemias and lymphomas in thymus-derived lymphocytes. Cancer 36: 1348–1353 (1975).

12 Mills, B., Sen, L., and Borella, L.: Reactivity of antihuman thymocyte serum with acute leukemic blasts. J. Immun. 115: 103–107 (1975).

13 Mittal, K. K., Mickey, M. R., Singal, D. P., and Terasaki, P. I.: Serotyping for homotransplantation. XVIII. Refinement of microdroplet lymphocyte cytotoxicity test. Transplantation 6: 913–918 (1968).

14 Netzel, B., Rodt, H., Lau, B., Thiel, E., Haas, R. J., Dörmer, P., and Thierfelder, S.: Transplantation of syngeneic bone marrow incubated with leukocyte antibodies. II. Cytotoxic activity of anti-cALL globulin on leukemic cells and normal precursor cells in man. Transplantation 26: 157–161 (1978).

15 Pike, B. L. and Robinson, W. A.: Human bone marrow colony growth in agar-gel. J. cell Physiol. 76: 77–82 (1970).

16 Pinkel, D., Hernandez, K., Borella, L., Holton, C., Aur, R., Samoy, G., and Pratt, C.: Drug

dosage and remission duration in childhood lymphocytic leukemia. Cancer 27 247 252 (1971).
17 Ringelmann, R. W Opferkuch, W Röllinghoff, M und Loos, M Komplement messungen mit Hilf des Mikrotitersystems. Z. med. Mikrobiol. Immun. 154 329–332 (1969).
18 Rodt, H Thierfelder S Thiel, E. Gotze, D Netzel, B Huhn, D and Eulitz, M. Identification and quantitation of human T cell antigen by antisera purified from antibodies crossreacting with hemopoietic progenitors and other blood cells. Immunogenetics 2. 411–419 (1975)
19 Rodt, H Netzel, B., Thiel, E Jäger G., Huhn, D Haas, R J Gotze, D and Thierfelder S Classification of leukemic cells with T and O-ALL specific antisera in Thierfelder Rodt and Thiel, Immunological diagnosis of leukemias and lymphomas (Springer New York 1977).
20 Rodt, H., Netzel, B Thiel, E Schneider U Mmowada, J Haas, R J and Thierfelder S Characterisation of antigens on leukemic cells and cell lines using specific heterologous antisera in Peters, Protides of the biological fluids (Pergamon Press, Oxford 1978).
21 Schlossmann, S. F Chess, L., and Humphreys, R E Distribution of Ig-like molecules on the surface of normal and leukemia human cells. Proc. natn. Acad. Sci USA 73 1288–1292 (1976).
22 Schneider U and Schwenk, H U Characterisation of T and non-T cell lines established from children with acute lymphoblastic leukemia and non-Hodgkin-lymphoma after leukemic transformation, in Thierfelder Rodt and Thiel, Immunological diagnosis of leukemias and lymphomas (Springer New York 1977).
23 Schwenk, H. U and Schneider U Cell cycle dependency of T-cell marker on lymphoblasts. Blut 31 299–305 (1975).
24 Seligmann, M., B cell and T cell marker in lymphoid proliferations. New Engl. J Med. 290 1483–1491 (1974).
25 Sen, L. and Borella, L. Clinical importance of lymphoblasts with T markers in childhood acute leukemia. New Engl. J Med. 292 828–832 (1975).
26 Thiel, E., Dörmer P Roth, H., Huhn, D Bauchinger M., and Thierfelder S Quantitation of T-antigenic sites and Ig-determinants on leukemic cells by microphotometric immunoradioautography in Thierfelder, Rodt and Thiel, Immunological diagnosis of leukemias and lymphomas (Springer New York 1977).
27 Thiel, E., Rodt, H Netzel, B Huhn, D Wundisch, G. F Haas, R. J Bender-Götze, Ch., und Thierfelder S., T-Zell-Antigen positive, E-Rosetten negative akute Lymphoblasten leukämie. Blut 37 363–366 (1978).
28 Tsukimoto, U Wong, K. J and Lampkin, B. C. Surface markers and prognostic factors in acute lymphoblastic leukemia. New Engl. J. Med 294 245–248 (1976)

Received: March 3, 1978
Accepted. August 18, 1978

Prof Dr med R. J Haas,
Kinderklinik der Universität München,
Abteilung Hämatologie/Onkologie,
Lindwurmstrasse 4, D–8000 München 2

Acta haemat. 61 184–193 (1979)

Structural and Functional Characteristics of Hairy Cells

Felice Salsano Simonetta Pisarri-Salsano Maria Pia Ciancarelli Mauro Piantelli Libero Lauriola and Piero Musiani

Istituto di Patologia Speciale Medica I Università di Roma, and
Istituto di Anatomia ed Istologia patologica, Università Cattolica, Roma

Key Words. Hairy cells Chronic lymphocytic leukemia Surface immunoglobulins Surface Ig capping Immunoperoxidase Electron microscopy

Abstract. Morphological cytochemical immunological and ultrastructural studies were performed on peripheral blood mononuclear cells from a patient with hairy-cell leukemia. Immunofluorescence studies showed a very strong intensity of fluorescence and indicated that hairy cells had monoclonal surface membrane immunoglobulins (SmIg) actively produced by the cells. An unusual spontaneous SmIg redistribution induced by antibodies was also noted. Immunoultrastructural studies demonstrated that antibody-induced redistribution of SmIg on hairy cells is in form of a singular polar cap and that the cell membrane is rapidly cleaned of the complexes by endocytosis. The behavior of hairy cells regarding several membrane markers, mitogen stimulation and antibody-induced cytotoxicity suggests that hairy projections could represent the expression of a functional stage common to different lymphocyte subpopulations, or alternatively a marker of a peculiar subset of B lymphocytes.

Hairy-cell leukemia is an infrequent form of malignant lymphoreticular disorder in which the proliferating cells have peculiar cytochemical and morphological features [13] The nature of the hairy cell is controversial some authors [3–5 7–9 11 21] favor a B lymphocytic origin while other investigators suggest a monocytic or histiocytic derivation [12 16 27 29 31]

We have studied a case of hairy-cell leukemia using morphological cytochemical immunological and ultrastructural methods. Moreover in an attempt to better understand the nature of the leukemic hairy cell we have studied the production of surface membrane immunoglobulins (SmIg) and the fate of anti Ig-surface Ig complexes using immunofluorescence and immunoperoxidase electron microscopy We present evidence that immunoglobulins are actively

synthetized and can be redistributed on the membrane of hairy cells as on normal B lymphocytes.

Materials and Methods

Case History

A. A. V. 45-year-old man, was noted to have lymphocytosis during routine checkup. Physical examination showed massive splenomegaly. The hemoglobin was 10.9 g/dl and the white cell count 17 × 10⁹/l, with 18% neutrophils, 79% lymphocytes and 3% monocytes. The platelet count was 188 × 10⁹/l. The total serum protein was 8.1 g/dl. The blood smears and the bone marrow imprints showed typical large mononuclear cells with increased amounts of cytoplasm, most of which had numerous slender hair-like cytoplasmic projections. This was confirmed by phase microscopy. Acid phosphatase staining, including tartrate inhibition [31–33], was positive. Hairy-cell leukemia was then diagnosed.

For the transmission electron microscopy blood was immediately fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for 2 h using two different methods as reported by *Katayama and Schneider* [15].

Immunological Investigations

Peripheral blood mononuclear cells were isolated on Ficoll Hypaque density gradient [2]. The percentage of cells forming spontaneous rosettes with sheep red blood cells (E rosettes) or rosettes with ox erythrocytes sensitized with anti-ox IgG (EA ox rosettes) or with human O R₁R₁ erythrocytes coated with an anti-Rh IgG (EA human rosettes) was determined as previously described [22].

Membrane immunoglobulins were identified in an immunofluorescence test. Preparation of antisera against human μ, δ, γ, α, λ, κ chains and against F(ab')₂ fragment of human IgG, conjugation with fluorescein isothiocyanate (FITC) and incubation were performed as reported elsewhere [22, 24]. Before staining, mononuclear cells were always incubated overnight at 37 °C in serum-free medium and washed at the same temperature. Moreover to investigate whether the Ig found on the cell surfaces were actually synthetized by these cells, the same cells were treated with trypsin (30 min) and incubated in medium for 6 h as described by *Preud'Homme and Seligmann* [20]. In capping experiments the cells were incubated with the fluorescein-labelled antiserum at 4 °C for 30 min in the presence of sodium azide (1 mg/ml). This was followed by washing of the cells at 4 °C in a medium with and without sodium azide and incubation at 37 °C, and, in some experiments, at 4 °C, in humidified atmosphere with 5% CO_2 for 3, 5, 15, 30, 45 min. Microscopy and microphotography were performed as previously described [24]. For ultrastructural studies of SmIg distribution, cells were fixed overnight at 4 °C with 4% paraformaldehyde before exposure to rabbit antibodies against human Ig. On the other hand, to study surface Ig redistribution induced by antibodies (capping), fixation in 4% buffered paraformaldehyde was carried out after the exposure of the cells to antihuman Ig rabbit antibodies. Finally the cells were incubated for 30 min at 37 °C with the peroxidase-labelled [1] goat IgG antirabbit IgG.

The number of latex phagocytes was calculated by a method recently described by us [26]. Briefly 0.1 ml of latex particles (1.1 μm) coated with IgG was added to 5 × 10⁶ mononuclear cells in 1 ml RPMI 1640 containing 20% fetal calf serum. This mixture was incubated 90 min at 37 °C on a shaking table, washed twice in RPMI 1640 + 20% FCS and then once in RPMI 1640 + 1 mg/ml Na azide. Using this method, phagocytic cells, engulfed by latex particles, were easily distinguishable from the Fc receptor bearing cells showing the same particles only around the membrane. Antibody-dependent cell-mediated cytotoxicity (ADCC) was performed according to *Perlmann and Perlmann* [19], details were given elsewhere [17].

Blast cell transformation after stimulation with phytohemagglutinin (PHA), concanavalin A (ConA), pokeweed mitogen (PWM), and purified protein derivative (PPD) was performed on microculture plates, details of the procedure and doses of mitogens are described elsewhere [25].

Peripheral mononuclear cells were also tested by Dr. *Roberto Tosi*, with several Merrit B-cell alloantiserums in complement-dependent microcytotoxicity tests. The human antisera were obtained

from various sources as reported by *Halford et al* [30].

Results

The morphology (fig. 1) of the neoplastic cells from peripheral blood appeared similar to that reported by several authors [5 11 14 15 28]

The vast majority of cells (88%) was positive in immunofluorescence when stained with the pepsinic fragment of an antiserum directed against the $F(ab')_2$ fragment of human IgG (table I) Almost the same proportion of cells was stained positively by antisera monospecific for the human μ δ and λ chains. The intensity of fluorescence was very strong and a spontaneous redistribution of the fluorescence was noted in spite of 4 C incubation and 0.2% Na azide concentration Further evidence that the immunoglobulins present on the surface of the cell were produced by the cell was obtained in trypsinization experiments or preincubating the cells overnight at 37 C after these procedures the same pattern of fluorescence appeared.

Percentage values of several surface markers are reported in table I We found the Fc receptor on a large proportion of the peripheral blood lymphocytes using rosette formation tests with ox-sensitized erythrocytes (EA ox 63%) and with human IgG coated latex particles (EA latex 79%), while only 12% of the cells formed rosettes with sensitized human erythrocytes (EA human). The percentage of E rosette was very low (6%)

A small proportion (8%) of cells ingested latex particles coated with human IgG (table II) Results from mitogenic stimulation experiments represented about 50% of the normal values in our laboratory (table II).

Table I. Surface markers on peripheral blood mononuclear cells in hairy-cell leukemia

		A.A.V	Normal[1]
White blood cells		17 × 10⁹/l	4–10 10⁹/l
Hairy cells,		72	–
Surface membrane	F(ab₁)₂	88	13.3 ± _5
immunoglobulin, %	M	8	8 1 ± 3 4
	D	80	9.0 ± 2.3
	G	1	1.0 ± 0.3
	K		8.2 ± 2 6
	L	85	3 6 ± 2.1
Rosette-forming	E	6	63 1 ± 4 1
cells,	IgG EA (ox)	63	16.3 ± 1.2
	IgG EA (human)	12	10.3 ± _6
	IgG EA (latex)	79	14.2 ± _4

[1] Peripheral blood mononuclear cells.

[2] Values indicate means ± SD for our laboratories.

Table II. Immunologic and functional studies on peripheral blood mononuclear cells in hairy-cell leukemia

	A.A.V	Normal[1, 2]
Stimulation with		
Phytohemagglutinin, cpm	44 161	70–100,000
Concanavalin A, cpm	56,877	75–170 000
Pokeweed mitogen, cpm	34 116	50– 81,000
Purified protein derivative, cpm	19,_31	_8– 46,000
Cytotoxicity induced by		
Phytohemagglutinin,	28 1	20–40
Antibodies,	37.3	45–70
Latex phagocytosis,	8	3–20

Peripheral blood mononuclear cells.

[2] Range values, for our laboratories, i normal subjects.

Spontaneous transformation in lymphocytes from control subjects was 731 ± 309 cpm

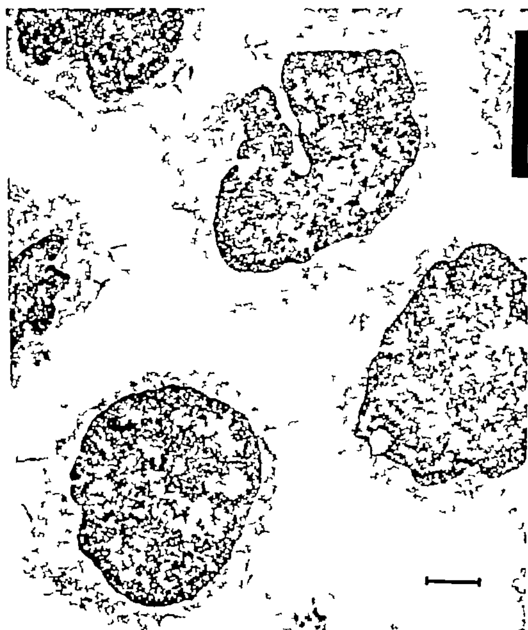


Fig. 1 Electron micrograph of hairy cells. Numerous slender finger-like processes are seen on the cell surface. Multiple small blebs can be observed within the microvilli. The heterochromatin is condensed around the nuclear membrane. The line represents µm

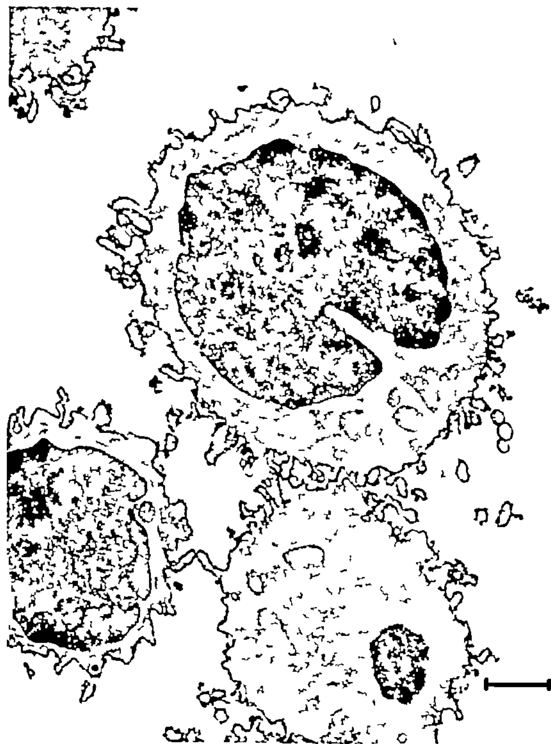


Fig. 2. Electron micrograph of hairy cells showing a strong diffuse labell ng of all villous membrane The cells were fixed in 4 / parafor maldehyde and then incubated with rabbit antibodies against human IgM After the second incubation with peroxidase-coupled goat IgG against rabbit immunoglobulin, the peroxidase ctı lty was revealed following the method of *Graham nd Karnovsky* [10] The line represents 2 m

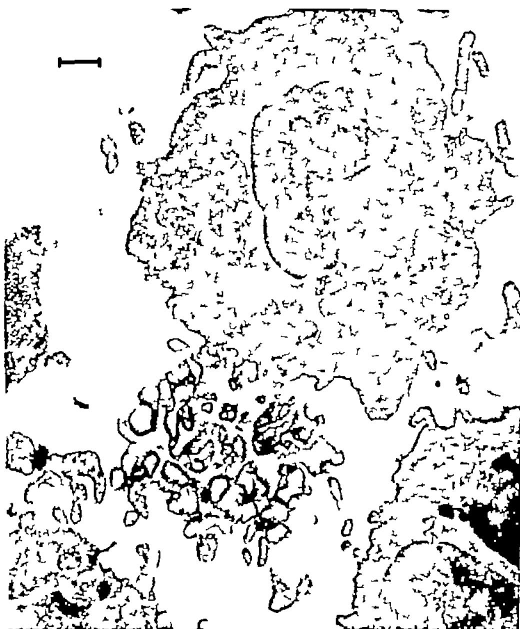


Fig. 3. Electron micrograph of hairy cell capping Ig-anti-Ig complexes. Note the marked constriction, forming the uropod, in the area surrounding the cap. All the label is in the cap area over the uropod. The line represents 1 μm.

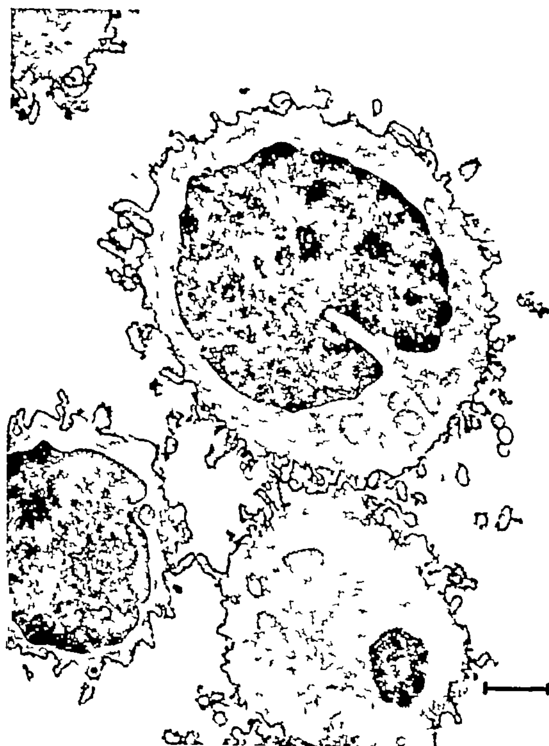


Fig. 2. Electron micrograph of hairy cells showing a strong diffuse labelling of all villous membrane. The cells were fixed in 4 paraformaldehyde and then incubated with rabbit antibodies against human IgM. After the second incubation with peroxidase-coupled goat IgG against rabbit immunoglobulin, the peroxidase activity was revealed following the method of *G aham and K rnovsky* [10]. The line represents μm.

PHA-induced cytotoxicity and antibody-dependent cell-mediated cytotoxicity were investigated the results expressed as cytotoxic index (CI = 28 1 and 37 4 %, respectively) even if lower than in normal subjects, demonstrated that hairy cells exibit a certain degree of activity (table II) Light and electron microscopy studies of SmIg showed that SmIg-anti-Ig complexes, uniformely distributed around the cell membrane, including that of the hairy projection (fig. 2), at the starting time were rapidly redistributed to a polar cap where numerous villous projections were found (fig. 3). Finally the SmIg-anti-Ig complexes were internalized by endocytosis (fig. 4). This pattern of redistribution was similar to that observed in normal B lymphocytes studied as control the difference consisted in the amounts of the cap-forming cells and in the rapidity of the capping slightly affected by low temperature and presence of sodium azide

Peripheral mononuclear cells obtained from the patient showed positive reactions with the Merrit B cell alloantisera.

Discussion

The determination of surface markers on mononuclear peripheral cells of our patient indicated that the vast majority of cells has on its surface immunoglobulins, actively produced by the cells. The restricted expression of heavy (μ, δ) and light (λ) chains of surface immunoglobulins strongly suggests that hairy-cell leukemia represents a clonal B cell proliferation as chronic lymphocytic leukemia (CLL) [6, 24]

A high percentage of cells had receptors for the Fc fragment of the IgG moreover the simultaneous investigation for membrane immunoglobulins and EA latex rosettes showed that both markers are present on the same neoplastic cell.

As recently observed by *Naeim et al.* [18] hairy cells from our patient too showed positive reactions with the Merrit B cell alloantiserums. Merrit B cell alloantigens have been detected on chronic lymphocytic leukemia cells, B-type lymphoid cell lines and peripheral blood B lymphocytes but not on Fc-positive SmIg-negative mononuclear cells [30]

The immunological features so far described do not allow a clear-cut distinction between hairy and CLL cells.

Immunofluorescence and ultrastructural studies clearly demonstrated that antibody induced redistribution of SmIg is in the form of a singular polar cap, as it generally happens with B lymphocytes and not with monocytes. During the capping process all the membrane became smooth with the exception of the capped zone where finger-like processes and numerous microvilli could be detected. In the last stage of this phenomenon the cell became round and its membrane appeared completely smooth. This ultrastructural finding is probably related to the observed rapid cleaning of the complexes by endocytosis. Finally the peculiar SmIg capping of hairy cells, not influenced by low temperature and by inhibitors of energy metabolism, may be the result of an atypically high membrane fluidity related to a decreased viscosity of the lipid bilayer This may reflect an altered cholesterol/phospholipid ratio. CLL cells do not exhibit these properties, therefore we attribute to hairy-cell leukemia a peculiar position in the field of B cell lymphoproliferative disorders

In this study we produce evidence that hairy cells are B lymphocytes in other re-

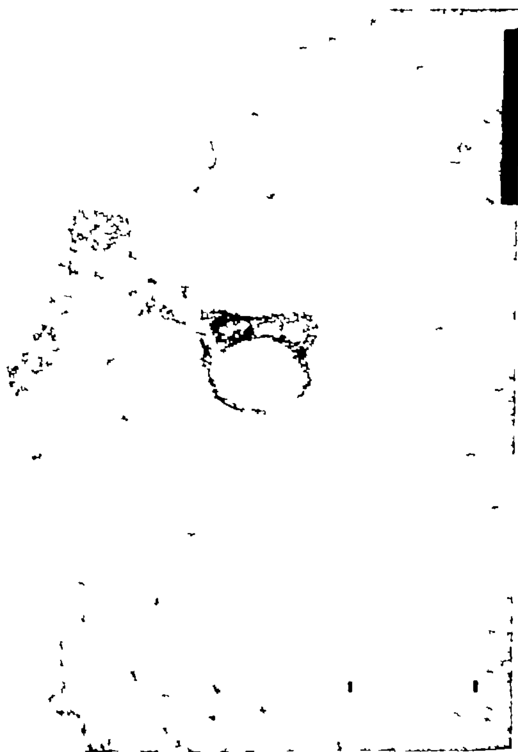


Fig 4. Electron micrograph showing a typical pattern of the last stage of the surface Ig redistri bution the surf ce Ig-anti-Ig complexes endocytosis. The line represents 1 μm.

PHA-induced cytotoxicity and antibody-dependent cell-mediated cytotoxicity were investigated the results expressed as cytotoxic index (CI = 28 1 and 37 4 /₀, respectively), even if lower than in normal subjects, demonstrated that hairy cells exhibit a certain degree of activity (table II) Light and electron microscopy studies of SmIg showed that SmIg-anti-Ig complexes, uniformely distributed around the cell membrane, including that of the 'hairy' projection (fig. 2), at the starting time were rapidly redistributed to a polar cap where numerous villous projections were found (fig. 3). Finally the SmIg-anti-Ig complexes were internalized by endocytosis (fig. 4). This pattern of redistribution was similar to that observed in normal B lymphocytes studied as control the difference *consisted in the amounts of the cap-*forming cells and in the rapidity of the capping slightly affected by low temperature and presence of sodium azide.

Peripheral mononuclear cells obtained from the patient showed positive reactions with the Merrit B cell alloantisera.

Discussion

The determination of surface markers on mononuclear peripheral cells of our patient indicated that the vast majority of cells has on its surface immunoglobulins, actively produced by the cells. The restricted expression of heavy (μ, δ) and light (λ) chains of surface immunoglobulins strongly suggests that hairy-cell leukemia represents a clonal B cell proliferation as chronic lymphocytic leukemia (CLL) [6 24]

A high percentage of cells had receptors for the Fc fragment of the IgG moreover the simultaneous investigation for membrane immunoglobulins and EA latex rosettes showed that both markers are present on the same neoplastic cell.

As recently observed by *Naeim et al.* [18] hairy cells from our patient too showed positive reactions with the Merrit B cell alloantiserums. Merrit B cell alloantigens have been detected on chronic lymphocytic leukemia cells, B-type lymphoid cell lines and peripheral blood B lymphocytes but not on Fc-positive SmIg-negative mononuclear cells [30]

The immunological features so far described do not allow a clear-cut distinction between hairy and CLL cells.

Immunofluorescence and ultrastructural studies clearly demonstrated that antibody induced redistribution of SmIg is in the *form of a singular* polar cap, as it generally happens with B lymphocytes and not with monocytes. During the capping process all the membrane became smooth with the exception of the capped zone where finger-like processes and numerous microvilli could be detected. In the last stage of this phenomenon the cell became round and its membrane appeared completely smooth. This ultrastructural finding is probably related to the observed rapid cleaning of the complexes by endocytosis. Finally the peculiar SmIg capping of hairy cells, not influenced by low temperature and by inhibitors of energy metabolism, may be the result of an atypically high membrane fluidity related to a decreased viscosity of the lipid bilayer This may reflect an altered cholesterol/phospholipid ratio. CLL cells do not exhibit these properties, therefore we attribute to hairy-cell leukemia a peculiar position in the field of B cell lymphoproliferative disorders.

In this study we produce evidence that hairy cells are B lymphocytes in other re



Fig. 4. Electron micrograph show ng a typical pattern of the last stage of the surface Ig redistri bution the surface Ig anti-Ig complexes endocytosis. The line represent 1 μm.

with hairy cells. New Engl. J. Med. 296, 882 (1977).

19 Perlmann, P. and Perlmann, H., Contactual lysis of antibody-coated chicken erythrocytes by purified lymphocytes. Cell. Immunol. 1 300–315 (1970).

20 Preud'Homme, J. L. and Seligmann, M., Surface bound immunoglobulins as cell marker in human lymphoproliferative diseases. Blood 40: 777 794 (1972).

21 Rubin, A. D. Douglas, S. D. Chessin, L. N. Glade, P. R., and Dameshek, W. Chronic reticulolymphocytic leukemia-reclassification of 'leukemic reticuloendotheliosis' through functional characterization of circulating mononuclear cells. Am. J. Med. 47 149–162 (1969).

22 Salsano, F. Monoclonal membrane immunoglobulins on the surface of chronic lymphocytic leukemia, hairy cell, leukemia and lymphoma cells. Proc. Meet. on 'Recent Advances in Clinical Immunology' Florence 1977 (in press, 1978).

23 Salsano, F. Frøland, S. S., Natvig, J. B. and Mandelli, F. Chronic lymphocytic leukemia cells: studies on the effect of drug treatment on different lymphocytes subpopulations. Scand. J. Immunol. 5 1185–1190 (1976)

4 Salsano, F. Frøland, S. S., Natvig, J. B., and Michaelsen, T. E., Some idiotype of B lymphocytes membrane IgD and IgM. Formal evidence for monoclonality of chronic lymphocytic leukemia cells. Scand. J. Immunol. 3 841–846 (1974).

25 Salsano, F. Mannella, E., De Rossi, G. Fontana, L. Pisarri-Salsano, S. Villa Santa, M. F. Popolazioni linfocitarie terapia nella leucemia linfoide cronica. Prog. Med. 32. 317 323 (1976)

26 Salsano, F. Pisarri-Salsano, S., Ciancarelli, M. P. Cocco, P. and Amadei, G. The latex IgG rosette test for the simultaneous detection of phagocytizing cells and of cells with F receptor for IgG (submitted for publication, 1978).

27 Scheinberg, M., Brenner, A. L., Sullivan, A. L., Cathcart, E. S. and Katayama, L. The heterogeneity of leukemic reticuloendotheliosis, 'hairy cell leukemia. Evidence for its monocytic origin. Cancer 37 1302 1307 (1976).

28 Schnitzer B. and Kass, L. Hairy-cell leukemia. A clinico-pathologic and ultrastructural study. Am. J. clin. Path. 61 176–187 (1974).

29 Seshadri, R. S. Brown, E. J. and Zipursky A., Leukemic reticuloendotheliosis. A failure of monocyte production. New Engl. J. Med. 295. 181 184 (1976).

30 Walford, R. L., Ferrara, G. B., Gatti, R. A., Leibold, W. Thompson, J. S., Mercuriali, F. Gossett, R., and Naeim, F. New groups and segregant series among B-cell alloantigens of the Merrit system. study of leukemia cells, peripheral B-cells, and lymphoblastoid cell lines. Scand. J. Immunol. 6. 393–402 (1977).

31 Yam, L. T. Castoldi, G. L., Garvey, M. B. and Mitus, W. J. Functional cytogenetic and cytochemical study of the leukemic reticulum cells. Blood 32 90–101 (1968).

32 Yam, L. T. Li, C. Y. and Finkel, H. F. Leukemic reticuloendotheliosis: the role of tartrate-resistant acid phosphatase in diagnosis and splenectomy in treatment. Archs intern. Med. 130 248–256 (1972).

33 Yam, L. T. Li, C. Y. and Lam, K. W. Tartrat resistant acid phosphatase isoenzyme in the reticulum cells of leukemic reticuloendotheliosis. New Engl. J. Med. 284 357–360 (1971)

Received: February 1 1978
Accepted: August 10, 1978

Dr. Piero Musiani,
Istituto di Anatomia patologica,
Università Cattolica S. Cuore,
Via Pineta Sacchetti, 644,
I-00168 Roma (Italy)

ports some authors demonstrated that the hairy cells have some properties of the cells belonging to the monocytic line. On the basis of these observations we can hypothesize that microvilli or finger like processes represent the expression of a functional stage common to different lymphocyte subpopulations, or alternatively a marker of a peculiar subset of B cells.

Acknowledgement

The authors gratefully acknowledge the expert technical assistance of Mr *Paolo Baldassarri*

References

1 Avrameas, S. and Ternynck, T., Peroxidase labelled antibody and Fab conjugated with enhanced intracellular penetration. Immunochemistry *8* 1175–1179 (1971)

2 Bøyum A. Separation of leukocytes from blood and bone marrow Scand. J clin. Lab. Invest. *21* suppl. 97 p. 7 (1968).

3 Catovsky D Pettit, J E Galetto, J., Okos, A., and Galton, D A. G The B-lymphocyte nature of the hairy cell of leukaemic reticuloendotheliosis. Br J Haemat. 6. 29–37 (1974).

4 Catovsky D Pettit, J E., Galton, D A. G Spiers, A. S. D and Harrison, C. V Leukaemic reticuloendotheliosis ('hairy cell leukemia) a distinct clinico-pathological entity Br J Haemat. *26* 9–27 (1974).

5 Debusscher L. Bernheim, J L. Collard-Ronge' E. Govaerts, A. Hooghe, R., Lejeune, F J Zeicher M and Stryckmans, A. Hairy cell leukemia functional, immunologic, kinetic, and ultrastructural characterization. *Blood 46 495–507 (1975)*

6 Fu, S. M Winchester R. J Feizi, T Welzer P D and Kunkel M G Idiotypic specificity of surface immunoglobulin and the maturation of leukemic bone-marrow-derived lymphocytes Proc. natn. Acad. Sci. USA *71* 4487–4490 (1974).

7 Fu, S. M Winchester R. J Rai, K. R., and Kunkel, H G., Hairy cell leukemia. proliferation of a cell with phagocytic and B-lymphocyte properties. Scand. J Immunol *3* 847–851 (1974)

8 Golde, D W Quan, S G and Cline, M. J Hairy cell leukemia. *In vitro* culture studies. Ann. intern. Med. *85* 78–79 (1976).

9 Golde, D W., Saxon, A and Stevens, R. H., Macroglobulinemia and hairy-cell leukemia. New Engl. J Med. *296*. 9? 93 (1977).

10 Graham, R. C. and Karnovsky M. J Glomerular permeability Ultrastructural cytochemical studies using peroxidases or protein tracers. J exp. Med *124* 1123–1133 (1966)

11 Haak, H. L., De Man, J C. H. Hymans, W Knapp, W and Speck, B., Further evidence for the lymphocytic nature of leukaemic reticuloendotheliosis (hairy-cell leukemia). Br J Haemat. 27 31–38 (1974).

1 Jaffe, E. S. Shevach, E. M., Frank, M M., and Green, I Leukemic reticuloendotheliosis: presence of a receptor for cytophilic antibody Am J Med. 57 108–114 (1974).

13 Katayama, I and Finkel, H E., Leukemic reticuloendotheliosis. A clinicopathologic study with review of the literature Am. J Med. *57* 115–1 6 (1974).

14 Katayama, I., Li, C. Y and Yam, L. T Ultrastructural characteristics of the 'hairy cells of leukemic reticuloendotheliosis. Am. J Path. *67* 361–366 (1972).

15 Katayama, I and Schneider G B., Further ultrastructural characterization of hairy cells of leukemic reticuloendotheliosis. Am. J Path. *86* 163–174 (1977)

16 King, G W Hurtubise, P E. Sagone A. L., Jr Lobuglio, A. F and Metz, E. N Leukemic reticuloendotheliosis. A study of the origin of the malignant cell Am. J Med. *59* 411–416 (1975)

17 Larrson, A., Pisarri-Salsano ,S Øhlander C., and Natvig, J B Destruction of dextran-coated target cells by normal human lymphocytes and monocytes. Induction by a human anti-dextran serum with IgG antibodies restricted to the IgG II subclass. Scand. J Immunol. *4* 241–.52 (1975).

18 Naeim, F Gossett, T and Walford, R. L., Reactivity of the Merrit B-cell alloantiserums

Surface Marker Study

Lymphoid cells were separated from heparinized venous blood by differential gradient sedimentation using the Ficoll-Hypaque technique [2]. T cells were identified by spontaneous rosette formation with sheep erythrocytes (E) [16] with slight modification. The lymphoid cell preparation (10^6 cells in 0.25 ml absorbed fetal calf serum) was mixed with an equal volume of 1 % E suspension. The mixture was kept at room temperature for 15 min and then centrifuged at 300 g for 5 min. After about 18 h incubation at 4 C, the pellet was gently resuspended and the number of cells with three or more red cells adherent (rosettes) were determined. A minimum of 200 lymphoid cells were counted to compute the percent of T lymphocytes. B cells were detected by immune rosettes (EAC rosettes) [1] with slight modification.

The mononuclear cells showed only small percentage of cells with B cell marker 3% and T cell marker 5% thus suggesting that Sézary cells in our patient lack the characteristics of both T and B cells. The surface marker study repeated 1 year later when peripheral smear did not show any Sézary cells, revealed 22% T cells and 3% B cells

Cytogenetic Study

Peripheral blood was cultured for 24 h without stimulation with phytohaemagglutinin Chromosome preparations were made according to the method of *Hungerford* [11]. Metaphases were stained for G banding. 8 out of 22 cells analyzed showed normal diploid karyotype while 8 cells ere hypodiploid with loss of one of chromosomes, 45 XY F (table I) In addition to these cells, few cells showed trisomy of G (47 XY+G) Marker chromosomes in A group and D group were present in tw cells

Table I. Karyotype analysis

Number of metaphases	Karyotype
8	46 XY
8	45 XY − F
5	47 XY + G (one cell with markers in A and D)
1	45 XY − G

The patient was put on oral chlorambucil 10 mg/day and prednisolone 40 mg/day for 2 months. The skin lesion regressed completely and abnormal cells disappeared from the peripheral blood. After 2 months, the patient was put on chlorambucil 2 mg/day H remained well with occasional recurrence of skin lesions. On 2. 8. 78, he developed pneumonia and expired. Post-mortem was not done.

Discussion

Among the T cell malignancies, mycosis fungoides (MF) and SS with primary cutaneous involvement constitute the major proportion [5 19] The disease may remain localized to the skin for some years ultimately leading to lymphadenopathy and hepatosplenomegaly

Whether Sézary cells belong to the lymphocytic or the monocytic series has been much disputed in the past. The results of recent studies are strongly in favour of their lymphoid nature. Also, th presence of T cell markers like receptors for sheep erythrocytes [12] reactivity with heterologous anti-T cell serum and lack of surface immunoglobulin have confirmed the T cell nature of Sézary cells with few exceptions [8, 10] Recently *Broder et al.* [3] identified Sézary cells as helper T cells by their capacity to regulate immunoglobulin synthesis from B cells. The acid phosphatase activity also favours T cell origin [9] The presence of *clones or grossly aneuploid cells in PHA* stimulated blood cultures [6] suggest that they are neoplastic cells and the fact that they are responsive to PHA is a clear indication that they belong to T lymphoid series. The absence of receptors for E rosette in the present patient and in a few previously reported series [4 10 0] raises the possibility that SS may be composed of two

Acta haemat. 61 194–197 (1979)

A Case of Sézary Syndrome with Non-Rosetting Cells and Unusual Cytogenetic Findings

S H Advani A G Khare J J Nadkarni A N Bhisey J S Nadkarni and G V Talvalkar

Tata Memorial Hospital and Cancer Research Institute, Tata Memorial Centre, Parel, Bombay

Key Words. Sézary syndrome T and B surface markers Chromosome

Abstract. A rare case of Sézary syndrome with typical clinical and haematological picture is described The absence of any lymphoid surface markers on Sézary cells and the consistent presence of distinct clone 45 XY F cytogenetic abnormality are very unusual features observed in the present case In spite of these peculiar findings, response to chlorambucil and prednisolone was excellent

The Sézary syndrome (SS) a primary cutaneous lymphoma, is characterized by circulating mononuclear cells with deeply folded or cerebriform nucleus. Cytogenetically it is associated with heteroploid cells with marker chromosomes [18] and with respect to cell surface properties, it has come to be regarded as one of the rare examples of human T cell neoplasia [13]

In this paper we report a case of SS with typical clinical and morphologic findings. Results of cytogenetic and surface marker studies are also discussed

Case Report

A 47 year-old Hindu male patient was seen in November 1976 with history of intractable pruritus, progressive hyperpigmentation and lichenification of the skin of 3 years duration. He was being treated with steroids for eczematous dermatitis. He had generalized lymphadenopathy but no hepatosplenomegaly A blood count showed a haemoglobin of 9.2 g/dl, WBC count of 52,400/µl with 70% mature looking atypical lymphocytes displaying some clefting of nucleus, 20% polymorphs, 6% eosinophils and 4% monocytes. The serum alkaline phosphatase was 2 sigma U/ml with serum creatinine 1.1 mg/dl and serum uric acid 4.9 mg/dl. Bone marrow aspiration and serum immunoelectrophoresis were normal. Histological examination of the skin biopsy revealed a few atypical reticulum cells beside focal collection of lymphocytes and a few plasma cells. This cellular infiltrate was seen in the perivascular region and in the upper dermis. The lymph node showed mainly appearance of dermatopathic lymphadenitis along with a few scattered large mononuclear cells in sinusoids and in the interfollicular region. Imprint of the lymph node showed a number of mononuclear cells with large deeply clefted or convoluted nuclei and scanty cytoplasm resembling Sézary cells.

Surface Marker Study

Lymphoid cells were separated from heparinized venous blood by differential gradient sedimentation using the Ficoll-Hypaque technique [2]. T cells were identified by spontaneous rosette formation with sheep erythrocytes (E) [16] with slight modification. The lymphoid cell preparation (10^6 cells in 0.25 ml absorbed fetal calf serum) was mixed with an equal volume of 1 / E suspension. The mixture was kept at room temperature for 15 min and then centrifuged at 300 g for 5 min. After about 18 h incubation at 4 C, the pellet was gently resuspended and the number of cells with three or more red cells adherent (rosettes) were determined. A minimum of 200 lymphoid cells were counted t compute the percent of T lymphocytes. B cells were detected by immune rosettes (EAC rosettes) [1] with slight modification.

The mononuclear cells showed only small percentage of cells with B cell marker 3% and T cell marker 5% thus suggesting that Sézary cells in our patient lack the characteristics of both T and B cells. The surface marker study repeated 1 year later when peripheral smear did not show any Sézary cells, revealed 22% T cells and 3% B cells.

Cytogenetic Study

Peripheral blood was cultured for 24 h without stimulation with phytohaemaglutinin. Chromosome preparations were made according to the method of *Hungerford* [11]. Metaphases were stained for G banding. 8 out of 22 cells analyzed showed normal diploid karyotype while 8 cells were hypodiploid with loss of one of chromosomes, 45 XY F (table I). In addition to these cells, few cells showed trisomy of G (47 XY+G). Marker chromosomes in A group and D group were present in two cells.

Table I. Karyotype analysis

Number of metaphases	Karyotype
8	46 XY
8	45 XY – F
5	47 XY + G (one cell hh markers in A and D)
1	45 XY – G

The patient was put on oral chlorambucil 10 mg/day and prednisolone 40 mg/day for 2 months. The skin lesion regressed completely and abnormal cells disappeared from the peripheral blood. After 2 months, the patient was put on chlorambucil 2 mg day. He remained well with occasional recurrence of skin lesions. On 2. 8. 78, he developed pneumonia and expired. Post-mortem was not done.

Discussion

Among the T cell malignancies, mycosis fungoides (MF) and SS with primary cutaneous involvement constitute the major proportion [5 19]. The disease may remain localized to the skin for some years ultimately leading to lymphadenopathy and hepatosplenomegaly.

Whether Sézary cells belong to the lymphocytic or the monocytic series has been much disputed in the past. The results of recent studies are strongly in favour of their lymphoid nature. Also the presence of T cell markers like receptors for sheep erythrocytes [14] reactivity with heterologous anti-T cell serum and lack of surface immunoglobulin have confirmed the T cell nature of Sézary cells with few exceptions [8, 10]. Recently *B oder et al* [3] identified Sézary cells as helper T cells by their capacity to regulate immunoglobulin synthesis from B cells. The acid phosphatase activity also favours T cell origin [9]. The presence of clones or grossly aneuploid cells in PHA stimulated blood cultures [6] suggest that they are neoplastic cells and the fact that they are responsive to PHA is a clear indication that they belong to T lymphoid series. The absence of receptors for E rosette in the present patient and in a few previously reported series [4 10 20] raises the possibility that SS may be composed of two

Acta haemat. *61* 194–197 (1979)

A Case of Sézary Syndrome with Non-Rosetting Cells and Unusual Cytogenetic Findings

S H Advani A G Khare J J Nadkarni A N Bhisey J S Nadkarni and G V Talvalkar

Tata Memorial Hospital and Cancer Research Institute, Tata Memorial Centre, Parel, Bombay

Key Words. Sézary syndrome   T and B surface markers   Chromosome

Abstract. A rare case of Sézary syndrome with typical clinical and haematological picture is described. The absence of any lymphoid surface markers on Sézary cells and the consistent presence of distinct clone 45 XY F cytogenetic abnormality are very unusual features observed in the present case In spite of these peculiar findings, response to chlorambucil and prednisolone was excellent.

The Sézary syndrome (SS), a primary cutaneous lymphoma, is characterized by circulating mononuclear cells with deeply folded or cerebriform nucleus. Cytogenetically it is associated with heteroploid cells with marker chromosomes [18] and with respect to cell surface properties, it has come to be regarded as one of the rare examples of human T cell neoplasia [13]

In this paper we report a case of SS with typical clinical and morphologic findings. Results of cytogenetic and surface marker studies are also discussed

Case Report

A 47 year-old Hindu male patient was seen in November 1976 with history of intractable pruritus, progressive hyperpigmentation and lichenification of the skin of 3 years duration. He was being treated with steroids for eczematous dermatitis. He had generalized lymphadenopathy but no hepatosplenomegaly A blood count showed a haemoglobin of 9.2 g/dl, WBC count of 52,400/μl with 70% mature looking atypical lymphocytes displaying some clefting of nucleus, 20% polymorphs, 6% eosinophils and 4% monocytes. The serum alkaline phosphatase was [illegible]2 sigma U/ml with serum creatinine 1.1 mg/dl and serum uric acid 4.9 mg/dl. Bone marrow aspiration and serum immunoelectrophoresis were normal. Histological examination of the skin biopsy revealed a few atypical reticulum cells beside focal collection of lymphocytes and a few plasma cells. This cellular infiltrate was seen in the perivascular region and in the upper dermis. The lymph node showed mainly appearance of dermatopathic lymphadenitis along with a few scattered large mononuclear cells in sinusoids and in the interfollicular region. Imprint of the lymph node showed a number of mononuclear cells with large deeply clefted or convoluted nuclei and scanty cytoplasm resembling Sézary cells.

12 Brouet, J.-C., Flandrin, G. and Seligman, M.: Indication of the thymus derived nature of the proliferating cells in si patients with Sézary syndrome. New Engl. J. Med. 289: 341-344 (1973).

13 Lutzner, M., Edelson, R., Schein, P., Green, I., Kirkpatrick, C. and Ahmed, A.: Cutaneous T-cell lymphomas: the Sézary syndrome, mycosis fungoides and related disorders. Ann. intern. Med. 83: 534-552 (1975).

14 Lutzner, M. A., Emerit, I. and Durepaire, R.: Cytogenetic, cytophotometric and ultrastructural study of large cerebriform cells of the Sézary syndrome and description of small cell variant. J. natn. Cancer Inst. 50: 1145-1162 (1973).

15 Prunieras, M.: DNA content and cytogenetic of the Sézary cell. Proc. Mayo Clin. 49: 548-552 (1974).

16 Stjernsward, J., Joodal, M., Vanky, F., Wigzell, H. and Sealy, R.: Lymphopenia and change in distribution of human B and T lymphocytes in peripheral blood induced by irradiation for mammary carcinoma. Lancet i: 1352-1356 (1972).

17 Taswell, H. F. and Winkelmann, R. K.: Sézary syndrome — malignant reticulemic erythroderma. J. Am. med. Ass. 177: 465-472 (1961).

18 Whang-Peng, J., Lutzner, M.; Edelson, R., and Kautsen, T.: Cytogenetic studies and clinical applications in patients with Sézary syndrome. Cancer 38: 861-867 (1976).

19 Winkelmann, R. K. and Linman, T. W.: Erythroderma with atypical lymphocytes (Sézary syndrome). Am. J. Med. 55: 192-198 (1973).

20 Wybran, J. and Fudenberg, H. H.: Thymus derived rosette forming cells in various human disease states: cancer, lymphomas, bacterial and viral infection and other diseases. J. clin. Invest. 52: 1026-1032 (1973).

21 Wara, D. W., Goldstein, A. L., Doyle, N. E., and Ammann, A. J.: Thymosin activity in patients with cellular immunodeficiency. New Engl. J. Med. 292: 70-74 (1975).

Received: July 18, 1978
Accepted: October 23, 1978

Dr. S. H. Advani, MD, Assistant Physician and Haematologist, Tata Memorial Hospital, Parel, Bombay-12 (India)

subgroups, one with T cell features and the other lacking both B and T cell characteristics. The cell type in our patient can be either null cells or T cells at an early stage of cell maturation This could be resolved by incubating cells with thymosin, which is able to induce E rosettes in committed precursors of T lymphocytes [21] Also the use of anti-T ALL sera for detection of T cell antigen would help to establish the exact nature of the cells in our patient

Cytogenetic data on a total of 21 SS patients has been reported so far [7 14 15] The range of modal chromosome number in 12 patients was 42–152. Markers were present in 10 patients while minute chromosomes were found in 4 patients. In the majority of the cases, the range was narrow but was rather broad in 7 patients *Whang-Peng et al* [18] did not observe clones in SS patients. In contrast, our material showed a distinct clone 45 XY F and a small clone 47 XY+G Since all the metaphases did not show good bands, it is difficult to identify the chromosomes involved. A few metaphases showed good quality bands and were found to be diploid.

The SS responds to radiotherapy but its effect is temporary Aggressive chemotherapy has been more devastating than the disease. However combined use of continuous low dose chlorambucil and steroid therapy have shown good results with disappearance of erythrodermia in our case This probably suggests that response to therapy is independent of surface receptors and cytogenetic abnormality The patient died of pulmonary infection Commonly development of lymphoma, fulminant leukaemic process or infection are the terminal features. Perhaps, decrease in T lymphocytes even after treatment predisposes to these complications.

References

1 Bianco, C., Patrick, R., and Nussenzweig, U A population of lymphocytes bearing membrane receptor for antigen-antibody complement complexes. J exp. Med. *132* 702–709 (1970).

2 Boyum, A.. Separation of leucocytes from blood and bone-marrow Scand. J clin. Lab Invest. *21* suppl., pp 97–106 (1968).

3 Broder S. Edelson, R. L., Lutzner M. A., Nelson, D L., MacDermott, R. P., Durm, M. E., Goldman, C. K. McAde, B. D and Waldmann, T A.. The Sézary syndrome – a malignant proliferation of helper T-cell. J clin. Invest. *58* 1297–1306 (1976).

4 *Braylon, P., Variakojis, D and Yachnin, S.* The Sézary syndrome lymphoid cells, abnormal surface properties and mitogen responsiveness. Br J Haemat. *31* 553–564 (1975).

5 Clendenning, W E. Brecher G and Van Scott, E. J.. Mycosis fungoids relationship to malignant cutaneous reticulosis and Sézary syndrome. Archs Derm. *89* 785–792 (1964).

6 Crossen, P R. Mellor J L., Finlay A. G Robert, R. B. M Vincent, P C., and Gunz, F W The Sézary syndrome – cytogenetic studies and identification of the Sézary cell as an abnormal lymphocyte Am. J Med. *50* 4–34 (1971)

7 Dewald, G Spurbeck, J L., and Vitek, H. A.. Chromosomes in a patient with Sézary syndrome. Mayo Clin. Proc. *49* 553–557 (1974).

8 Edelson, R. L. Kirkpatrick, C H., Shevach, E. M *Schein, P S. Smith, R. W* Green, I and Lutzner M Preferential cutaneous infiltration by neoplastic thymus derived lymphocytes morphologic and functional studies. Ann. intern. Med. *80* 685–692 (1974).

9 Flandrin, G and Brouet, J C The Sézary cell cytologic, cytochemical and immunologic study Proc. Mayo Clin. *49* 575–583 (1974)

10 Goldstone, A. H Cawley J C Roberts, S. O Leventine, A., and Barker C R A case of small cell Sézary's syndrome with null cell features. J clin. Path. *29* 848–851 (1976)

11 Hungerford, D A.. Leucocyte cultures from inocula of whole blood and preparation of metaphase chromosomes by treatment with hypotonic KCl. Stain Technol. *40* 333–340 (1965).

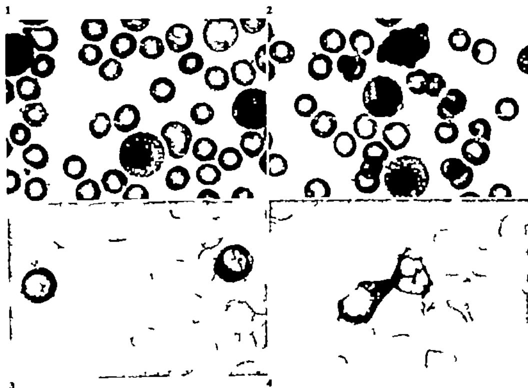


Fig. 1. Eosinophilic pseudo-Pelger-Huet cell with two blast cells. Peripheral blood. May-Grunwald Giemsa. × 800

Fig. 2. As figure 1 – note vacuoles in pseudo-Pelger Huet eosinophil

Fig. 3. Two pseudo-Pelger Huet cells filled with PAS-positive granules. PAS. No nuclear stain. × 800.

Fig. 4. Coarse granular and diffuse PAS positivity in pseudo-Pelger Huet eosinophil. Note normal nuclear segmentation and PAS diffuse positivity in the neutrophil granulocyt. Blasts are negative PAS. No nuclear stain. 800.

phadenopathy was present. Laboratory data disclosed hemoglobin 7.1 g/dl hematocrit 20.6%, erythrocytes 2.21 10⁶ l reticulocytes 60 10⁹ l, platelets 20 10⁹ l and leukocytes 17 10⁹ l differential count revealed, neutrophils 6%, eosinophils 10%, monocytes ?%, lymphocytes 14%, promyelocytes ?% and blast cells 66%. 72% of the eosinophils had round nucleus with clumped chromatin, 16% had an indented nucleus and 12% had bilobed nucleus, so that 'average lobe index' of eosinophils [31] was 1.12. These cells were considered to have the pseudo-Pelger-Huet anomaly. The eosinophils with round and indented nuclei could be easily distinguished from eosinophilic promyelocytes or myelocytes because they had relatively low nuclear-cytoplasmic ratio, heavily condensed chromatin and because they lacked azurophil granules. Many eosinophils had vacuoles in the nucleus and cytoplasm. Neutrophils had no nuclear abnormality. Bone marrow smears were rich of blast cells with myeloid: erythroid ratio of 98.2. Bone marrow eosinophilic cells were 10%, all of them had round pyknotic nuclei-like eosinophils observed in the peripheral blood. Except for the low platelet count reported above, the screening tests for evaluation of hemostasis (fibrinogen level, bleeding time, prothrombin time and partial thromboplastin time) were normal.

While the hematological data were unchanged, the patient general condition grew worse in

Acta haemat. *61* 198–202 (1979)

Eosinophilic Acquired Pelger-Huët Anomaly in Acute Myeloblastic Leukemia

A Cytochemical Study

M. Chilosi, V. Fossaluzza and F. Tosato

Istituto di Anatomia ed Istologia Patologica (Primario: Prof. *B. Antoci*) e Divisione Medica IV (Primario: Prof. *A. Bisaro*), Ospedale Civile di Udine, Generale Regionale, Udine

Key Words. Eosinophils · Pelger Huët anomaly · Myeloblastic leukemia

Abstract. Decreased nuclear segmentation and coarse chromatin only in mature eosinophils was seen in a patient with acute myeloblastic leukemia. These eosinophils were considered to have acquired Pelger Huët anomaly. On cytochemical study they displayed a strong PAS-positive reaction.

The Pelger Huët anomaly [12, 22] is a dominant hereditary disorder of granulocytes characterized by incomplete segmentation of nuclei and abnormal condensation of nuclear chromatin.

The acquired or pseudo-Pelger Huët anomaly has been found associated with a variety of conditions [1, 3–4, 5, 7, 9, 11, 14, 16, 23, 24, 26–29]. It seems rather frequent in myeloproliferative disorders [25] and is often observed in preleukemia [18]; thus it is considered a marker of a an abnormal marrow clone. In particular this anomaly strongly suggests the diagnosis of myeloid leukemia when it is present in unclassified acute leukemias [9, 16]. Usually both neutrophils and eosinophils have decreased nuclear segmentation and coarse chromatin.

In 1973 *Kay et al.* [15] described 2 cases of myeloproliferative disorders with pseudo-Pelger Huët anomaly of the eosinophils only. Their report remained unique. We present a similar case of pseudo-Pelger Huët anomaly confined to eosinophils in a patient with acute myeloblastic leukemia. A detailed cytochemical study of these cells was performed.

Case Report

A 23-year-old Italian man was admitted on October 29, 1976, with weakness, fatigue and pain in the left upper quadrant of the abdomen. He had been previously admitted 2 years before for acute otitis. On that occasion no anomaly of leucocytes was noted.

On physical examination he was in poor general condition with marked pallor, dyspnea and tachycardia. Blood pressure was 130/70 mm Hg. Ecchymoses in the left knee, chest and perianal region and painful ulcers on the gums and genitals were noted. The liver was palpated 3 cm and the spleen 4 cm below the costal margins. No lym-

Acta haemat. 61: 203–208 (1979)

Serum Folate Binding Capacity in Leukemias, Liver Diseases and Pregnancy

R. Corrocher, L. M. Bambara, M. L. Pachor, D. Biasi, A. Stanzial and G. De Sandre
Istituto di Patologia Medica II (Direttore: Prof. G. De Sandre), Università di Padova, Sede di Verona, Verona

Key Words. Folate · Leukemia · Acute hepatitis · Cirrhosis · Pregnancy · Protein binding

Abstract. Total and unsaturated folate binding capacity (TFBC, UFBC) have been measured in sera of selective groups of patients to study the role of cell turnover, cell necrosis and the effect of pregnancy in determining their concentrations in blood. The mean value of TFBC in 35 normal sera was 151 ± (SD) 53 pg/ml with a saturation of 88%. The TFBC was raised in chronic granulocytic leukemia (CGL), in acute hepatitis, in cirrhosis, and in pregnancy (third trimester). The normal mean value of TFBC was found in chronic lymphocytic leukemia (CLL) and in the first trimester of pregnancy. The mean UFBC in the normal sera was 19 ± 18 pg/ml. In all the pathological conditions studied the mean UFBC was significantly greater than normal and it was particularly high in CGL (85 ± 78 pg/ml).

Recently *Colman and Herbert* [1976] described a method for determination of the total folate binding capacity (TFBC) and the unsaturated folate binding capacity (UFBC) of human serum and measured them in the sera of normal subjects and in patients with uremia, cirrhosis and pregnancy conditions known to be associated with an increase in serum of UFBC.

In the present study UFBC and TFBC using a slightly modified technique have been measured in groups of patients with leukemia and acute or chronic hepatic damage to determine whether increased leukocyte or liver cell turnover might affect the serum concentration of the folate-binding protein.

Materials and Methods

Sera were obtained from 35 normal volunteers (19 males and 16 females), aged from 22 to 41 years, 15 patients with chronic granulocytic leukemia (CGL), 8 patients with chronic lymphocytic leukemia (CLL), 15 patients with acute hepatitis (Australia antigen positive), 18 patients with hepatic cirrhosis, and 41 pregnant women (21 in the *first trimester* and 20 *in the third trimester*) none of whom had received prophylactic folic acid therapy.

The blood was obtained approximately 2 h after breakfast by venipuncture, serum was removed within 2 h and stored at –20 °C for not more than 14 days before assay.

Reagents. The acidic and alkaline solutions used to buffer the sera were those used by *Colman and Herbert* [1976]; the buffering capacity was in the pH range 2.6–11.8 [*Britton and W.*

17 Leitner S. J and Gugelot, P C., New observations and studies on familial nuclear anomaly of leukocytes (Pelger Huët). Ned. Tijdschr Geneesk. 82 3953–3962 (1938).
18 Linman, J W and Saarni, M. I.. La sindrome preleucemica. Agg. in Emat. 12 376–386 (1975).
19 Mitus, W J et al.. Cytochemical studies of glycogen contents of lymphocytes in lymphocytic proliferations. Blood 13 748–756 (1958).
20 Rosse, W F and Gurney C. W.. The Pelger Huët anomaly in three families and its use in determining the disappearance of transfused neutrophils from the peripheral blood. Blood 14 170–186 (1959).
21 Park, B H. Dolen, J and Snyder B.. Defective chemotactic migration of polymorphonuclear leukocytes in Pelger Huët anomaly Proc. Soc. exp Biol. Med. 155 51–54 (1977).
22 Pelger K.. Demonstratie van een paar zeldzaam voorkomende typen van bloedlichaampjes en bespreking der patienten. Ned. Tijdschr Geneesk. 72 1178 (1928).
23 Phillips, B. A., Yarbro, J W and Konlon, G S. Acquired Pelger Huët anomaly in erythroleukemia. J Surg. Oncol. 2. 407–411 (1970)
24 Pusic, G and Teodori, S. Anomalia nucleare di Pelger Huët e pseudo-Pelger Haematologica 42 527–546 (1957).
25 Saarni, M. I. and Linman, J W.. Myelomonocytic leukemia. disorderly proliferation of all marrow cells. Cancer 27 1221–1230 (1971).
26 Seewann, H. L.. Terminal acute leukemia with pseudo-Pelger phenocopy in IgG plasmacytoma. Wien. med. Wschr 127 283–284 (1977).
27 Shanbrom, E. Collins, Z., and Miller S.. Acquired' Pelger Huët cells in blood dyscrasias. Am. J med. Sci. 240 732–737 (1960).
28 Shanbrom, E. and Tanaka, K. R.. Acquired Pelger Huët granulocytes in severe myxedema. Acta haemat. 27 289–293 (1962).
29 Stahl, A., Papy M. C., Muratore, R., Mongin, M. et Olmer J.. Erythromyélose avec pseudo-syndrome de Pelger Huët et anomalies chromosomiques complexes. Nouv Revue fr Hémat. 5 879–882 (1965)
30 Stobbe, H. und Jorke, D.. Befunde an homozygoten Pelger Merkmalsträgern. Schweiz. med. Wschr 95 1524–1529 (1965).
31 Taylor R. L. Pelger Huët anomaly and megaloblastic anemia. Am. J clin. Path. 60: 932–935 (1973).
32 Yam, L. T Li, C. Y., and Crosby W H. Cytochemical identification of monocytes and granulocytes. Am. J clin. Path. 55 283–290 (1971).

Received. April 4 1978
Accepted. September 20, 1978

Dr V Fossaluzza, Divisione Medica IV
Ospedale Civile di Udine, Generale Regionale,
via G Pieri, I-33100 Udine (Italy)

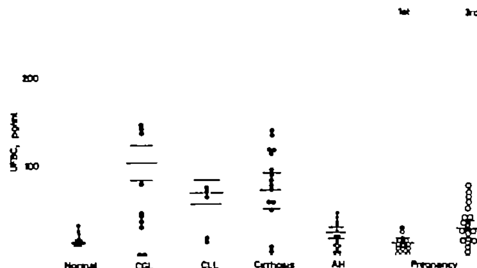


Fig. 3. Values of TFBC in serum of the patients studied (mean ± SD).

Table I. Statistic analysis of the results obtained in the different groups of patients

	Normal	CGL	CLL	Cirrhosis	Acute hepatitis	Pregnancy 1st	Pregnancy 3rd
TFBC, pg/ml							
Number	35	15	8	18	15	21	20
Mean	150.6	207.3	166.0	225.7	265.4	183.6	247.8
SD	± 53.1	± 76.2	± 54.4	± 117.4	± 70.7	± 58.7	± 119.9
p		< 0.01	NS	< 0.05	< 0.0005	NS	< 0.001
UFBC, pg/ml							
Number	35	15	8	18	15	21	20
Mean	19.5	85.0	57.5	49.8	32.1	31.9	62.9
SD	± 18.2	± 78.4	± 46.3	± 31.6	± 28.4	± 22.1	± 57.5
p		< 0.001	< 0.01	< 0.001	< 0.02	< 0.05	< 0.002

ford 1937] Norit A charcoal was prepared in distilled water (5 g/100 ml), and mixed with an equal volume of bovine albumin (Sigma) solution (1 g/100 ml in distilled water). Radioactive folic acid (pteroylglutamic acid – ^{3}H PteGlu, specific activity 37 Ci/mmol) was supplied by the Radiochemical Centre, Amersham, England. Its radiochemical purity was greater than 98% by paper chromatography; the elution on gel chromatography (Sephadex G-200) gave only one radioactive peak in the position of marker nonradioactive folic acid. It was diluted in normal saline and kept at –70 °C.

Folate Assay. Serum folate assay was performed using a radioisotopic method (Schwarz Mann Folate Radioassay Kit ^{125}I).

Determination of TFBC and UFBC. The method used is based on the denaturation of the serum binder at low pH and absorption of endogenous folate with coated charcoal before adding exogenous ^{3}H PteGlu to the serum alkalinized back to pH 7.4 [*Colman and Herbert* 1976]. The two modifications used here are: (1) an aliquot of serum used for determination of UFBC was absorbed at pH 7.4 with albumin-coated charcoal (ACC) before addition of ^{3}H PteGlu to remove free endogenous folate which might compete with ^{3}H PteGlu for protein binding as suggested by *Colman and Herbert* [1976]; (2) activated charcoal was coated with albumin (as above) instead of hemoglobin.

Chromatographic Studies. 1 ml of the final radioactive supernatant (for TFBC or UFBC determination) was eluted from Sephadex G-200 column (1.0 × 40 cm) previously equilibrated with phosphate-buffered saline (pH 7.4) and calibrated with blue dextran, hemoglobin, ovalbumin, β-lactoglobin and ^{3}H-PteGlu. 50 1-ml fractions were collected and the radioactivity of each fraction measured in a liquid scintillation counter (Packard), corrected for the quenching and plotted against the elution volume.

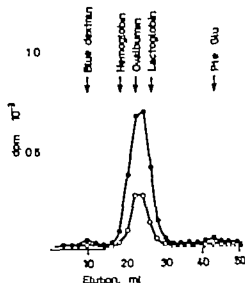


Fig. 1. Chromatographic profile of TFBC (●) and UFBC (O) incubated with ^{3}H PteGlu eluted from Sephadex G-200.

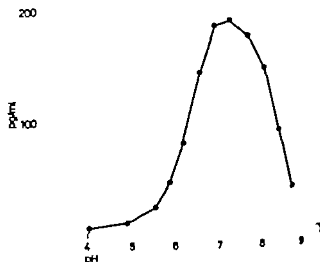


Fig. 2. Dependence of the folate binding on the pH: correlation between the pH of the serum and the total binding capacity for folic acid (pg/ml).

Results

Both the unsaturated and total folate binding proteins from either normal or CGL sera, eluted completely from Sephadex G-200 in the same position, immediately after ovalbumin, suggesting a molecular weight of approximately 42,000 (fig. 1). The binding capacity of serum was maximum within the pH range 6.5–8.0 (fig. 2).



Fig. 3. Values of TFBC in serum of the patients studied (mean ± SD).

Table I. Statistic analysis of the results obtained in the different groups of patients

	Normal	CGL	CLL	Cirrhosis	Acute hepatitis	Pregnancy	
						1st	3rd
TFBC, pg/ml							
Number	35	15	8	18	15	21	20
Mean	150.6	207.3	166.0	225.7	265.4	183.6	247.8
SD	± 53.1	± 76.2	± 56.4	± 117.4	± 70.7	± 58.7	± 119.9
P		< 0.01	NS	< 0.05	< 0.0005	NS	< 0.001
UFBC, pg/ml							
Number	35	15	8	18	15	21	20
Mean	19.5	85.0	57.5	49.8	32.1	31.9	62.9
SD	± 18.2	± 78.4	± 46.3	± 31.6	± 28.4	± 22.1	± 57.5
P		< 0.001	< 0.01	< 0.001	< 0.02	< 0.05	< 0.002

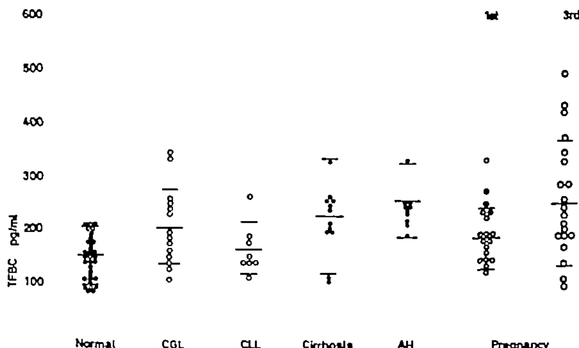


Fig. 4. Values of UFBC in the serum of the patients studied (mean ± SE).

After exposure to pH 4 and subsequent absorption with ACC, no folate could be measured in serum. The length of the exposure to both acidic solution or ACC up to 30 min did not modify the result of TFBC or UFBC.

TFBC and UFBC in normal serum. The results are given in figures 3 and 4 and analyzed statistically in table I. Each result is the mean of triplicate assays on a single serum. The mean TFBC of 35 normal sera was 151 ± 53 pg/ml (range 89–257 pg/ml). No significant difference was found between the sera of males and females and no correlation existed between the serum folate value and the TFBC. The mean normal UFBC was found to be 19.5 g/ml ranging from 66.2 pg/ml to immeasurably low levels (fig. 4). The mean saturation was therefore 132/151 = 88.4%.

TFBC and UFBC in CGL and CLL. In 15 sera from untreated patients with CGL, the mean TFBC was significantly raised compared to the normal group. In 7 (47%) of the 15 cases the TFBC was above the upper limit of normal (peak value 362 pg/ml). UFBC in CGL was also significantly greater than normal. On the other hand, among the 8 patients with CLL the mean TFBC was not significantly different, whereas the mean UFBC was higher than normal.

TFBC and UFBC in Cirrhosis. In these 18 sera the mean TFBC and UFBC were both significantly raised. There was no correlation between TFBC or UFBC with SGOT and SGPT or with serum folate or serum albumin concentrations.

TFBC and UFBC in Acute Hepatitis. In all but one of the sera from these 15 patients the TFBC exceeded the normal range

the mean TFBC, 265 ± 71 pg/ml was significantly greater than normal. On the other hand, the mean UFBC was also higher than normal (table I). Again, no correlation has been found between TFBC or UFBC with SGOT and SGPT or with serum bilirubin concentration.

TFBC and UFBC in Pregnancy. The mean value of TFBC in sera of women during the first trimester of pregnancy was slightly raised but the value was elevated in the third trimester ($p < 0.001$). However even in the first group, 5 of 21 patients (24 /) had a TFBC above the normal range. The mean UFBC in the two groups were both significantly raised. No correlation was found between the level of serum folate and the amount of binder in pregnancy.

Discussion

The results here confirm the findings of *Colman and Herbert* [1976] that it is possible reproducibly to measure the TFBC and UFBC in human serum. The results demonstrate a significant increase of TFBC in sera from patients with CGL whereas in sera from patients with CLL the TFBC was normal. These findings suggest that the myeloid line of cells is one possible source of the binder. The protein in CGL serum has a molecular weight of 42,000 which coincides with the MW of the binder found in the leukocytes and other tissues [*Rothenberg and da Costa*, 1976; *Colman and Heryert*, 1976] from where it may be released [*da Costa and Rothenberg*, 1976]. In both CGL and CLL the mean values of UFBC were greater than normal ($p < 0.001$ and $p < 0.01$ respectively), results which remain obscure since no correlation was found between the level of UFBC and the serum folate concentration.

On the other hand, the raised level of TFBC found here in sera of patients with acute hepatitis might be due to cell necrosis since liver cells are known to contain different folate binders, free in the cytosol or bound to nuclei and mitochondria [*Corrocher et al.*, 1974, 1976; *Zamierowski and Wagner*, 1977].

Cirrhosis represents a more complicated situation in which cell regeneration and necrosis often coexist. TFBC was also increased in patients with cirrhosis with a folate saturation on average lower than normal. These findings differ to some extent from those of *Colman and Herbert* [1976] who found the mean TFBC to be increased in cirrhosis but not significantly above normal. The discrepancy may be due to the difference in the severity of cirrhosis: our patients showed an increase in serum hepatic transaminases (mean twofold above normal) and cirrhosis was in general, in a severe uncompensated state. There was, however, no correlation between TFBC and serum enzyme concentration in the individual patient.

The level of TFBC was also greater than normal in the third trimester of pregnancy. The folic acid binding protein has previously been found to be increased in pregnancy and in women taking oral contraceptives [*Rothenberg and da Costa*, 1976], demonstrating that the presence of the binder in pregnancy is due to a new synthesis of the protein probably induced by hormones.

The folate-binding protein in serum has a greater affinity for folic acid than for the physiological form of folate in serum (methyltetrahydrofolate). Methyltetrahydrofolate does bind to this protein albeit with less af

finity than folic acid [*Rothenberg and da Costa* 1976]. Therefore, hot exogenous folic acid may displace methyltetrahydrofolate. If that is correct, it is uncertain why the exchange involves only a small fraction of endogenous folate since TFBC is almost ten times greater than UFBC in normal serum. It seems possible that more than one endogenous folate is bound to the protein: one easily displaced by hot folic acid at neutral or alkaline pH, the other removed only after exposure to low pH.

In conclusion, the results obtained here demonstrate that leukocytes and liver cells are the possible sources of serum folic acid binding protein from where it may be released into plasma. Estrogenic hormones may induce the synthesis of the protein and may cause an increased serum level of the binder in pregnancy or in women taking oral contraceptives. It is unlikely that this binder is involved in the transport of methyl tetrahydrofolate in plasma but it may play a role in the folate metabolism within the cells.

References

Britton, H. T. S. and Welford, G.: Standardisation of some buffer solution at elevated temperatures. J. Chem. Soc., Lond., part 2, pp. 1848–1852 (1937).

Colman, N. and Herbert, V.: Total folate binding capacity of normal human plasma, and variation in uremia, cirrhosis and pregnancy. Blood *48:* 911–921 (1976).

Corrocher, R.; De Sandre, G.; Pachor, M. L., and Hoffbrand, A. V.: Hepatic protein binding of folate. Clin. Sci. mol. Med. *46:* 551–554 (1974).

Corrocher, R.; Pachor, M. L.; Bambara, L. M.; Perini, A., and De Sandre, G.: Folate binding proteins in human digestive system. Rend. Gastroent. *8:* 29–3 (1976).

Da Costa, M. and Rothenberg, S. P.: Studies with the folate binding proteins in chronic granulocytic leukaemia cells. Br. J. Haemat. *34:* 581–587 (1976).

Hampers, C. L.; Streiff, R.; Nathan, D. C.; Snyder, D. and Merrill, S. P.: Megaloblastic hematopoiesis in uremia and in patients on long term hemodialysis. New Engl. J. Med. *276:* 551–564 (1967).

Herbert, V.: Aseptic addition method for *Lactobacillus casei* assay of folate activity in human serum. J. clin. Path. *19:* 12–16 (1976).

Rothenberg, S. P. and da Costa, M.: Folate binding proteins and radioassay for folate. Clin. Haematol. (1976).

Zamierowski, M. M. and Wagner, C.: Identification of folate binding protein in rat liver. J. Biol. Chem. *252:* 933–938 (1977).

Zetter, A. and Duly, P. E.: New evidence for a binding principle specific for folates as a normal constituent of human serum. Clin. Chem. *20:* 1313–1319 (1974).

Received, July 7, 1978
Accepted, September 27, 1978

Dr. R. Corrocher Roberto,
Istituto di Patologia Medica IIa,
Università di Padova,
Policlinico di Borgo Roma,
I-37100 Verona (Italia)

Acta haemat. 61: 209–215 (1979)

Auditory Involvement in Thalassemia Major[1]

S. De Virgiliis, F. Argiolu, G. Sanna, G. Cornacchia, P. Cossu, A. Cao, V. Mallardi and P. Puxeddu

Clinica Pediatrica, Clinica Otorinolaringoiatrica, Università di Cagliari, Cagliari

Key Words. Hearing defect · Iron overload · Thalassemia major · Serum ferritin

Abstract. The auditory function of 75 children affected by homozygous β^0-thalassemia, managed with a low transfusion scheme and treated irregularly with low doses of desferrioxamine, and of 75 controls were examined. In 12 patients a mild bilateral conductive hearing impairment due to bony hypertrophy and/or adenoid hypertrophy was found. In 43 cases a moderate monolateral or bilateral sensory-neural hearing loss at high frequencies with recruitment phenomenon was observed. Ferritin levels were determined in a randomly chosen group of these patients with (14) and without hearing loss (11). In the subjects with sensory-neural hearing loss the mean ferritin levels were significantly higher than in those with no hearing defect. There was no obvious relation between sensory-neural damage on the one hand and Hb levels and unit of blood transfused on the other. The results of this study suggest that iron overload could be a cause of damage in the high frequency elements of the auditory mechanism. Intermittent hypoxia and slow 8th nerve compression due to bony hypertrophy as causes of auditory involvement are also discussed.

There is little information on auditory involvement in thalassemia major. In 1972, *Logothetis et al.* [12] found progressive sensory-neural hearing loss in 2 patients aged 12 and 18 years. *Mosciaro and Borgatti* [19] showed a conductive hearing deficiency dependent on rhinopharynx involvement with Eustachian tube dysfunction in 10 patients out of 25 examined. *Hazell and Modell* [6] observed an elevated frequency of ear, nose and throat (ENT) involvement, particularly bony hypertrophy of the turbinates with Eustachian tube dysfunction and tonsillar and adenoid hypertrophy. Turbinate hypertrophy was seen to be more frequent and pronounced in the low transfusion regimen group. They also found hearing deficiency for frequencies 0.5–2–4 kHz ranging from 10 to 40 dB in 11 cases out of 25 examined.

[1] This work was supported by grants from the Assessorato Igiene e Sanità Regione Sarda, Sindacato Lavoratori Bancari and CNR contract number 77.00626.86.

In 1975 our study group found a mono or bilateral moderate sensory neural hearing loss at high frequencies (4–6–8 kHz) in 14 out of 20 patients affected by β^0-thalassemia major [13] The present investigation was undertaken to determine (a) the extent to which the sensory neural hearing mechanism is affected in thalassemia major and (b) the correlationships between sensory neural hearing loss on one hand and iron overload and/or chronic hypoxia on the other

Table I. Hearing impairment in 75 thalassemia major patients and 75 controls

	Thalassemia major patients	Controls
Normal[1]	20	68
Conductive defect	12	7
Sensory-neural defect	43	–

[1] Hearing pure tone at 20 dB was considered the threshold for pathological hearing loss.

Subjects and Methods

The patients studied included 43 females and 32 males between 3 and 13 years of age, with homozygous β^0-thalassemia. The diagnosis of homozygous β^0-thalassemia was made on the basis of hemoglobin electrophoresis and globin chain synthesis studies according to *Kan et al.* [8]. 18 of these cases had been splenectomized. The patients *included in this study had had transfusion limited* to a minimum allowing Hb levels to fall as low as 5–7 g/dl before the next transfusion. Prior to this study the average pretransfusion Hb ranged from 5 to 8 g/dl. Only in the last 2 years had the patients been transfused with sufficient packed red cells every 3–4 weeks to maintain their pretransfusion Hb levels to above 9 g/dl.

Chelation therapy with desferrioxamine has been administered irregularly and at low doses except in the last 2 years when it has been given intramuscularly in doses of 750 mg/day from 3 to 9 years and in doses 1 g/day over 9 years of age for 6 consecutive days every week.

Individual testing was performed by measurement of pure tone air and bone conduction thresholds and Bekesy tracing (when considered necessary). The hearing test was carried out with the Grason-Stadler-Bekesy type audiometer model

Table II. Subjects with conductive hearing loss

Subjects	Sex	Age	Turbinate hypertrophy	Tonsillary hypertrophy	Adenotonsillitis	Pure tone audiogram[1]	Treatment required
D F	F	5		+++	+++	25	adenotonsillectomy
C.M	M	6		+++	+++	20	
V.M.	M	5		+++	+++	30	
Z.M.C.	F	6	+	++	+++	15	
T.M.	M	6	+	++	++	10	
F.M.T	F	7		+++	+++	25	
G D	F	5		+++	+++	20	
P M.	M	7	++	+		15	
C.G L.	M	5	++			15	
C.M.L.	F	6	+			15	
C.G	M	8	++	+++	+++	25	adenotonsillectomy
S.F	F	12	+	+		20	

[1] Average loss in dB, both ears, over 250, 500, 1,000, 2,000 Hz.

1701. Hearing pure tone at 20 dB or more was considered the threshold for pathological hearing loss.

As a control, 75 normal subjects closely matched for age, (4–12 years) origin and socio-economical status were also examined using the same methods.

Table III. Average sensory-neural hearing loss in dB over 3–4–6–8 kHz

	Frequency Hz			
	3,000	4,000	6,000	8,000
Average loss in dB	24.2	24.3	24.7	26.9
Number of patients affected	7/43	20/43	40/43	43/43

Serum ferritin was assayed in 25 randomly selected patients with (14 cases) and without (11 cases) sensory-neural hearing loss. Serum ferritin concentration was measured in venous blood as ferritin protein using the immunoradiometric assay described by *Addison et al.* [1] modified by Miles [1, 7 11, 21] (immunoradiometric Kit Ramco Laboratories Houston, Tex.). Systematic monitoring of SGOT and SGPT levels had been carried out every 30 days in each patient.

Results

Turbinate hypertrophy tonsillar hypertrophy and recurrent adenotonsillitis was seen in 40, 31 and 28% of the cases examined, respectively These were more fre

Table IV Serum ferritin level and average sensory-neural hearing loss in dB over 4–6–8 kHz in homozygous β^0-thalassemic patients

Subjects without hearing loss				Subjects with sensory-neural hearing loss									
subjects	sex	age, years	Serum ferritin, ng/dl	subjects	sex	age, years	Serum ferritin, ng/dl	right ear dB			left ear dB		
								4,000 Hz	6,000 Hz	8,000 Hz	4,000 Hz	6,000 Hz	8,000 Hz
S.R.	M	9	1,300	C.P.P	F	5	2,400	10	10	15	10	20	25
A.D	M	6	80	D.R.	M	5	79	15	20	20	20	20	20
V.S.	M	10	2,000	C.L.	M	11	4,160	10	15	20	15	25	30
F G.	F	8	40	C.R.	F	8	1,600	15	15	15	10	20	35
C.R.	F	8	520	T.R.	M	5	5,200	10	25	20	15	15	15
P.L.	M	5	1,150	S.M.C.	F	10	2,300	10	20	25	15	20	20
C.O.L.	M	6	2,560	M.A.	F	6	2,080	25	25	30	25	20	20
P.M.A.	F	8	424	M.S.	M	6	2,960	15	20	30	15	20	20
M.M.O	F	10	1,500	M.P	F	7	1 440	10	20	20	15	20	20
M.M.	M	6	800	A.P	F	12	128	25	20	25	10	10	10
P.M.D.	F	3	440	C.A.	M	7	1,300	20	40	35	10	30	35
				B.L.	M	8	1 440	20	25	25	15	20	20
				S.G.	F	7	1,584	20	20	30	15	20	20
				U.E.	F	7	3,200	25	30	35	20	20	20
Mean			983.09				2,133 64						
SD			804 78				1 411.99						
P			< 0.05										

In 1975 our study group found a mono or bilateral moderate sensory neural hearing loss at high frequencies (4–6–8 kHz) in 14 out of 20 patients affected by β^{o}-thalassemia major [13]. The present investigation was undertaken to determine: (a) the extent to which the sensory neural hearing mechanism is affected in thalassemia major and (b) the correlationships between sensory neural hearing loss on one hand and iron overload and/or chronic hypoxia on the other.

Table I. Hearing impairment in 75 thalassemia major patients and 75 controls

	Thalassemia major patients	Controls
Normal[1]	20	68
Conductive defect	12	7
Sensory-neural defect	43	–

[1] Hearing pure tone at 20 dB was considered the threshold for pathological hearing loss.

Subjects and Methods

The patients studied included 43 females and 32 males between 3 and 13 years of age, with homozygous β^{o}-thalassemia. The diagnosis of homozygous β^{o}-thalassemia was made on the basis of hemoglobin electrophoresis and globin chain synthesis studies according to *Kan et al.* [8]. 18 of these cases had been splenectomized. The patients included in this study had had transfusion limited to a minimum allowing Hb levels to fall as low as 5–7 g/dl before the next transfusion. Prior to this study the average pretransfusion Hb ranged from 5 to 8 g/dl. Only in the last 2 years had the patients been transfused with sufficient packed red cells every 3–4 weeks to maintain their pretransfusion Hb levels to above 9 g/dl.

Chelation therapy with desferrioxamine has been administered irregularly and at low doses except in the last 2 years when it has been given intramuscularly in doses of 750 mg/day from 3 to 9 years and in doses 1 g/day over 9 years of age for 6 consecutive days every week.

Individual testing was performed by measurement of pure tone air and bone conduction thresholds and Bekesy tracing (when considered necessary). The hearing test was carried out with the Grason-Stadler Bekesy type audiometer model

Table II. Subjects with conductive hearing loss

Subjects	Sex	Age	Turbinate hypertrophy	Tonsillary hypertrophy	Adenotonsillitis	Pure tone audiogram[1]	Treatment required
D.F	F	5		+++	+++	25	adenotonsillectomy
C.M	M	6		+++	+++	20	
V.M.	M	5		+++	+++	30	
Z.M.C.	F	6	+	++	+++	15	
T.M	M	6	+	++	++	10	
F.M.T	F	7		+++	+++	25	
G.D	F	5		+++	+++	20	
P.M.	M	7	++	+		15	
C.G.L.	M	5	++			15	
C.M.L.	F	6	+			15	
C.G	M	8	++	+++	+++	25	adenotonsillectomy
S.F	F	12	+	+		20	

[1] Average loss in dB both ears, over 250, 500, 1,000, 2,000 Hz.

1701 Hearing pure tone at 20 dB or more was considered the threshold for pathological hearing loss.

As control, 75 normal subjects closely matched for age, (4–12 years) origin and socio-economical status were also examined using the same methods.

Table III. Average sensory-neural hearing loss in dB over 3-4-6-8 kHz

	Frequency Hz			
	3,000	4,000	6,000	8,000
Average loss in dB	24.2	24.3	24.7	26.9
Number of patients affected	7/43	20/43	40/43	43/43

Serum ferritin was assayed in 25 randomly selected patients with (14 cases) and without (11 cases) sensory-neural hearing loss. Serum ferritin concentration was measured in venous blood as ferritin protein using the immunoradiometric assay described by *Addison et al.* [1] modified by Miles [1, 7 11, 21] (Immunoradiometric Kit Ramco Laboratories Houston, Tex.). Systematic monitoring of SGOT and SGPT levels had been carried out every 30 days in each patient.

Results

Turbinate hypertrophy tonsillar hypertrophy and recurrent adenotonsillitis was seen in 40 31, and 28% of the cases examined, respectively These were more fre

Table IV. Serum ferritin level and average sensory-neural hearing loss in dB over 4-6-8 kHz in homozygous β^0-thalassemic patients

Subjects without hearing loss				Subjects with sensory-neural hearing loss									
subjects	sex	age, years	Serum ferritin, ng/dl	subjects	sex	age, years	Serum ferritin, ng/dl	right ear dB			left ear dB		
								4,000 Hz	6,000 Hz	8,000 Hz	4,000 Hz	6,000 Hz	8,000 Hz
S.R.	M	9	1,300	C.P.P	F	5	2,400	10	10	15	10	20	25
A.D.	M	6	80	D.R.	M	5	79	15	20	20	20	20	20
V.S.	M	10	2,000	C.L.	M	11	4 160	10	15	20	15	25	30
F.G.	F	8	40	C.R.	F	8	1,600	15	15	15	10	20	35
C.R.	F	8	520	T.R.	M	5	5,200	10	25	20	15	15	15
P.L.	M	5	1,150	S.M.C.	F	10	2,300	10	20	25	15	20	20
C.G.L.	M	6	2,560	M.A.	F	6	2,080	25	25	30	25	20	20
P.M.A.	F	8	424	M.R.	M	6	4,960	15	20	30	15	20	20
M.M.G.	F	10	1,500	M.P	F	7	1 440	10	20	20	15	20	20
M.M.	M	6	800	A.P	F	12	125	25	30	25	10	10	10
P.M.D.	F	3	440	C.A.	M	7	1,300	20	40	35	10	30	35
				B.L.	M	8	1 440	20	25	25	15	20	20
				S.G	F	7	1,584	20	20	30	15	20	20
				U.E.	F	7	3,200	25	30	35	20	20	20
Mean			983.09				2,133.64						
SD			804.78				1,411.99						
P			< 0.05										

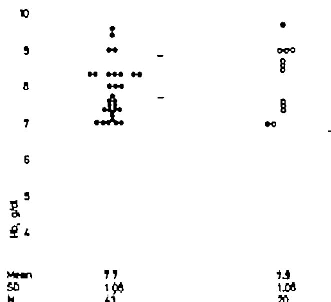


Fig. 1. Mean and SD Hb levels in homozygous thalassemic patients with (●) and without (O) sensory-neural hearing loss.

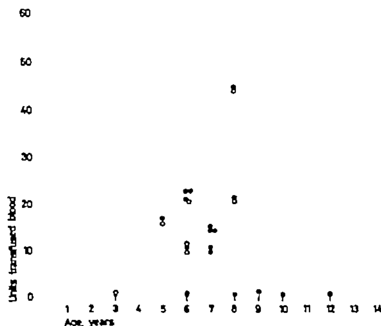


Fig. 2. Sensory-neural hearing loss in relation to the age and unit of transfused blood. Patients with sensory-neural hearing loss (●) and without (O).

quent in patients with conductive hearing loss.

In table I the type of hearing loss in 75 thalassemic patients and controls is shown.

In table II the ENT findings and mean hearing loss in both ears at 250 500 1 000 and 2,000 Hz frequencies in patients with conductive hearing loss can be seen.

55 out of 75 patients examined (73.33%) had hearing loss. In 12 patients this hearing deficiency was found to be bilateral and of the conductive type In the remaining 43

cases a moderate sensory-neural hearing de ficiency with the recruitment phenomenon at frequencies 3–4–6–8 kHz was observed. This defect was unilateral in 12 and bilateral in 31 patients.

Table III shows the mean sensory-neural hearing loss for frequencies 3–4–6–8 kHz in these patients. It can be seen that most patients have hearing loss at 6,000 and 8,000 Hz.

The loss in dB at high frequencies was never higher than 30–35 dB while at low and middle frequencies the hearing function was absolutely normal.

In 14 patients with hearing loss the se rum ferritin ranged from 79 to 5,200 ng/ml with a mean of 2,133.64 ± 1 411.99 (table IV). In 11 patients without hearing loss serum ferritin ranged from 40 to 2,560 ng/ml with a mean of 983.09 ± 804 78 (table IV). Although there was an overlap between the two groups, the differences between the mean was significant ($p < 0.05$).

As can be seen in figure 1 there was no evident relation between hearing loss on the one hand and annual mean Hb or units of blood received per year from the age of 31 ± 25 months on the other (fig. 2).

In figure 2, it can be seen that auditory deficiency at high frequencies does not increase with age.

Discussion

In thalassemia major patients with low transfusion regimen, a high incidence of both conductive and sensory neural hearing loss was found.

This frequency is similar to that found by *Hazell and Modell* [6] in patients with low transfusion regimen and much higher than that found by the authors in those with high transfusion regimen.

This defect was conductive in 12 cases and sensory-neural in 43 cases. These hear ing losses are not seriously handicapping as they seldom interfere with the perception of speech.

From the ENT findings in our patients, the conductive hearing loss is probably due to bony hypertrophy and/or adenoid hyper trophy according to *Hazell and Modell* [6]

In our study we have noted a significant correlation between hearing deficiency at high frequencies and serum ferritin levels. Since it has been established that serum fer ritin levels in patients with iron overload are correlated with the concentrations of iron in the liver our results suggest that a cause of auditory deficiency at high frequencies could be iron overload in the high frequency element of the auditory mechanism.

In contrast with the above results the auditory deficiency did not correlate with mean blood consumption and showed no progression with age [15] A possible ex planation for these contrasting results is that patients with Hb levels less than 10 g/dl have an increase of iron absorption in the intestine [4 17]

Evidence from postmortem material suggests that the iron increment received by this route is at least 3 mg/day A discrepancy between transfused iron load and the degree of involvement of endocrine glands in thalassemia major has also been found [15] Moreover it must be considered that stor age iron is also derived from ineffective er ythropoiesis and constant hemolysis. Another possible cause for the contrasting results previously discussed is the individual irregularity and variability of desferrioxamine administration carried out in our outpatients.

On the other hand it should be noted that *Bourguet et al* [2] found 9 cases of sensory neural and 2 cases of mixed hearing loss in a group of 43 subjects with primary hemochromatosis.

Another explanation for sensory-neural hearing loss could be chronic anoxia. In fact the basal turn of the cochlea (which records high frequencies), due to the high O_2 consumption and low capacity to use the anaerobic metabolism of the 'vascular stria is highly dependent on O_2 supply [3 9]

Animal studies have demonstrated that oxygen deprivation can result in irreversible damage to the auditory mechanism [5 10]

Furthermore, it should be noted that the congenital high frequency hearing impairment is actually considered a potential sequela to anoxia in the prenatal and perinatal period [14]

The high frequency hearing loss did not correlate with the mean Hb level. Moreover no alteration in sensory-neural hearing has been noted in patients with severe anemia (Hb < 5 g/dl) [20] However it should be noted that many patients were occasionally transfused when the Hb levels were as low as 3–4 g/dl. Severe intermittent hypoxia *may therefore have played* an additional role in the damaging process.

Another possible explanation could be the expansion of the temporal bone due to bone marrow hyperplasia, which in turn leads to a narrowing of the internal auditory canal and a progressive compression of the external fibres of the 8th nerve [18] It should be noted that with this type of slow compression the hearing defect is not necessarily associated by vestibular involvement.

In conclusion, our study shows that children with β^0 thalassemia major treated with low transfusion regimen and inadequate iron chelation can have conductive or sensory-neural hearing impairment. This could be due to iron overload eventually associated with chronic hypoxia and/or slow 8th nerve compression.

References

1 Addison, G M. Beamish, M. R., and Hales, C. M.. An immunoradiometric assay for ferritin in the serum of the normal subjects and patients with iron deficiency and iron overload. J clin. Path. 25 326–329 (1972).

2 Bourguet, J., Bourel, M., Simon, M., et Le Huerou, Y.. Hémochromatose et surdité. Revue Oto-Neuro-Ophtal. 42 215–224 (1970).

3 Conti, A. and Borgo, M.. Behaviour of cytochrome oxidase activity in the cochlea of the guinea pig following acoustic stimolation. Acta oto-lar 58 321–330 (1964).

4 Erlanson, M. E., Walden, B., Stern, G Hilgarther M. W Wehman, J., and Smith, S. H. Studies in congenital haemolytic syndromes. IV Gastrointestinal absorption of iron. Blood 19 359–378 (1962)

5 Hall, J G.. On the neuropathological changes in the central nervous system following neonatal asphyxia. Acta oto-lar suppl. 118, pp. 331–338 (1963)

6 Hazell, J W P and Modell, C. B.. ENT complications in thalassaemia major J Lar Otol. 90 877–881 (1976)

7 Jacobs, A. Miller F., Worwood, M., Beamish, M R., and Wardrop, C. A.. Ferritin in the serum of normal subjects and patients with iron deficiency and iron overload. Br med. J iv 206–208 (1972).

8 Kan, Y W., Schwartz, E., and Nathan, D G Globin chain synthesis in the alpha-thalassaemia syndromes. J clin. Invest. 47 2515–2522 (1968)

9 Koide, Y Hando, R., and Yoshikawa, Y.. Distribution of some oxidizing enzymes in the cochlea. Acta oto-lar 58 344–354 (1964).

10 Lawrence, M., and Wever E. G Effect of oxigen deprivation upon the structure of the organ of Corti, A. med. Ass. Archs Otolar 55. 31–37 (1952).

11 Lipschitz, D. A. Cook, J. D., and Finch, C. A.: A clinical evaluation of serum ferritin as an index of iron stores. New Engl. J. Med. *290*: 1213–1216 (1974).

12 Logothetis, J. Constantoulakis, M.; Economidou, J. Stefanis, C., Hakas, P. Augoustaki, O.; Sofroniadou, K., Loewenson, R., and Bilek, M.: Thalassemia major (homozygous β-thalassemia). A survey of 138 cases with emphasis on neurologic and muscular aspects. Neurology Minneap. *22*: 294–304 (1972).

13 Mallardi, V.; Fera, G. ed Argiolu, F.: Indagini audiometriche in bambini affetti da thalassemia major. Comun. LXII Congr. Soc. Ital. Otorinolar. Pat. Cervicofacciale, Capri 1975.

14 Matkin, N. D. The child with a marked high frequency hearing impairment. Pediat. Clins N. Am. *15*: 677–690 (1968).

15 McIntosh, N.: Endocrinopathy in thalassaemia major. Archs Dis. Childh. *51*: 195–201 (1976).

16 Miles, L. E. M., Lipschitz, D. A.; Bieber, C. P. and Cook, J. D.: Measurement of serum ferritin by a 2-site immunoradiometric assay. Analyt. Biochem. *61*: 209–224 (1974).

17 Modell, C. B.: Management of thalassaemia major. Br. med. Bull. *32*: 270–276 (1976).

18 Morgenstein, K. M. and Manace, E. D.: Temporal bone histopathology in sickle cell disease. Laryngoscope *79*: 2172 (1969).

19 Moscaro, D., e Borgatti, L.: La funzione uditiva nel morbo di Cooley. Atti Giornate di Studio Microcitemia, Ferrara 1965, vol. I, pp. 184–186.

20 Saha, A. M., Arora, M. M. L., and Mehra, Y. N. Eight nerve function in severe anaemia. An electronystagmographic study. Ind. J. Oto-Lar. *27*: 134–140 (1975).

21 Siimes, M., Addiego, J. E., jr. and Dallman, P. R.: Ferritin in serum. Diagnosis of iron deficiency and iron overload in infants and children. Blood *43*: 581–590 (1974).

Received: February 23, 1978
Accepted: July 22, 1978

Prof. Antonio Cao, Clinica Pediatrica II, Università di Cagliari, Via Porcell, 1, I-09100 Cagliari (Italy)

Acta haemat. 61 216–221 (1979)

Synthesis of Alpha, Delta-Beta and Gamma Chains by Reticulocytes from Two Brothers Homozygous for Haemoglobin Lepore

Benedetta Luppis and Valerio Ventruto

Laboratory of Molecular Embryology CNR, Arco Felice, and Department of Hematology Service of Hematological Immunopathology and Genetics, Ospedale Cardarelli, Naples

Key Words. Haemoglobin Lepore Globin chain synthesis Globin chain imbalance Homozygous Lepore

Abstract. Globin chain synthesis has been investigated for the first time in 2 patients homozygous for haemoglobin Lepore, although the 2 brothers have the same haemoglobin genotype the severity of the diseases is very different. The purpose of this study was to try and find out the reason for the different severity in the clinical manifestations. In the 2 patients a different excess of α-chain synthesis was observed the higher excess being present in the subject carrying the more severe anaemia. This result strongly suggests that in homozygous haemoglobin Lepore disease, as in β thalassaemia, the degree of globin chain imbalance is responsible for the clinical manifestations.

Introduction

The term haemoglobin Lepore designates a class of haemoglobin variants with normal α-chains, and an abnormal chain which is a hybrid of δ- and β-chains ($\delta\beta$-chain). Like δ and β the abnormal chain of Hb Lepore contains 146 amino acids The N-terminal portion is identical to that of the δ-chain, while the C terminal sequence corresponds to that of the β-chain. This unusual $\delta\beta$-chain results from an unequal crossover between the δ- and β-chain genes during meiosis [1] Three types of Hb Lepore have been described, which differ in the position of the crossover Hb Lepore Boston [2] Hb Lepore Baltimore [7] and Hb Lepore Hollandia [3]

Patients heterozygous and homozygous for haemoglobin Lepore have clinical and haematological features very similar to those of patients heterozygous and homozygous for β-thalassaemia [9]

The similarity between these two conditions is thought to depend on the fact that the $\delta\beta$-chain of the Hb Lepore, like the β-chain of thalassaemia, is synthesized at a rate slower than the α-chain, causing an overall imbalance of globin chain synthesis, like in β-thalassaemia. This hypothesis has been supported by a study of a subject heterozygous for Hb Lepore [11]

In this paper we describe the properties of haemoglobin synthesized by the peripheral red blood cells of 2 brothers homozygous for haemoglobin Lepore. The reason we did this study was to find out why the 2 patients, genetically identical for the homozygous $\delta\beta$-gene, had different clinical manifestations. Our results confirm that in the Hb Lepore, as in thalassaemia, there is an imbalance in globin chain synthesis. In both patients an excess of α-chain was observed, partially compensated by an increase of γ-chain synthesis. The synthesis of γ-chain was higher in the less severe case (L. P.).

Patients, Materials and Methods

Subjects

Probands were 2 brothers, 13 (L. P.) and 5 (P. P.) years old, from Caserta, South Italy. Preliminary data on these 2 patients were already reported [9]. The clinical symptoms were those of mild Cooley anaemia and were of different severity in the 2 brothers. The older one showed mild haemolytic anaemia. H never needed transfusions. The liver was only moderately enlarged. The spleen was hard and 4 cm below the costal border. Icterus was constantly present. The younger brother showed more severe haemolytic anaemia. H was regularly transfused. The liver was 1 cm and the spleen, very hard, was 7 cm below the costal border. After this work was accomplished he was splenectomized with consequent increase in the red cell count. Both brothers had normal weight and height in respect to their age. Haematological data are reported in table I. Blood smear showed anisopoikilocytosis and target cells. The diagnosis of homozygous haemoglobin Lepore was easily made from the haemoglobin pattern of the 2 patients and their parents [9].

Technical Procedures

2 ml of red blood cells were washed in saline and incubated for 60 min at 37 °C under standard conditions [4]. ^{3}H *L*-valine (New England Nuclear, 0.78 Ci/mmol) was added at the concentration of 0.02 µmol/ml. After hypotonic lysis, the stroma-free haemolysate was prepared by centrifugation at 10,000 *g* for 20 min, globin was precipitated by acid acetone treatment, dissolved in water lyophilized and kept at − 20 °C. Globin chains were separated by chromatography on Whatman CM 32 cellulose column according to the procedure of *Clegg et al.* [5]. 1 ml of each fraction (2 ml) was added to 10 ml of scintillator (toluene/PPO/Triton X 100) and counted in Nuclear Chicago liquid scintillation spectrometer. The optical density of each fraction was determined with Gilford spectrophotometer.

^{14}C-labelled globin was prepared from the peripheral blood of patient with autoimmune haemolytic anaemia by incubating the red blood cells as described, with ^{14}C-*L*-valine (0.1 µmol of ^{14}C-*L*-valine/ml of incubation mixture). ^{14}C-*L*-valine was from New England Nuclear 0.25 Ci/mmol.

Results

The analysis of the haemoglobin synthetized *in vitro* in the presence of ^{3}H-*L*-valine by the peripheral red blood cells of 1 of the 2 patients (P. P.) is reported in figure 1. To confirm the identity of the tritiated chains,

Table I. Haematological data of the 2 brothers

	Age years	Hb g/dl	RBC 0.10⁹/l	MCV fl	Retic ‰	Retic 10⁹/l	Serum iron µg/dl	Bilirubin mg/dl
L. P.	13	13	4.2	72	38	160	135	6
P. P.	5	8	2.4	70	45	108	145	2.7

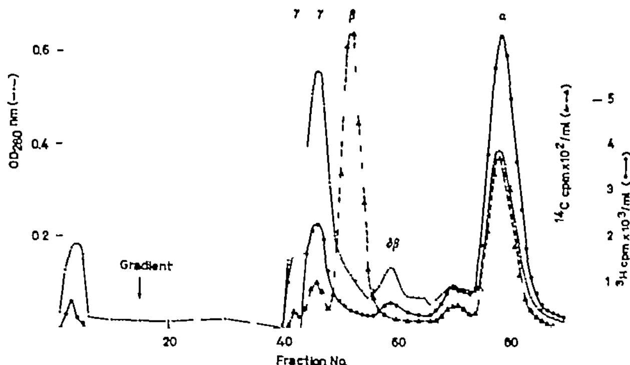


Fig. 1 Carboxymethyl cellulose column chromatography of 20 mg of globin synthesized by intact reticulocytes of P P in the presence of ^3H L-valine (O) 1 mg of normal adult globin, uniformly labelled with ^{14}C-L-valine was added as an internal standard before chromatography (△).

normal globin chains, uniformly labelled with ^{14}C L valine was added to the sample and then separated by chromatography on CM-cellulose. The α/non-α-chain ratio of the ^{14}C labelled globin was close to unity indicating that during the chromatography there is no preferential loss of one chain with respect to the other and that the peaks are not cross-contaminated by the other chains. At the same time the addition of ^{14}C labelled normal globin to the globin from the Hb Lepore subject, before chromatography gives a clear indication of the identity of the unknown chains. Figures 2 and 3 show the chromatographic analysis of the haemoglobin synthesized by the peripheral red blood cells of the 2 brothers. First of all this analysis confirms the diagnosis of homozygous Hb Lepore. The β and δ peaks are in fact absent and a δβ peak is present. The radioactivity incorporated in each chain is reported in table II Higher incorporation in P P is a reflection of higher degree of anaemia in this patient. The cpm incorporated in each chain were determined by adding the fractions (1 ml each) of each peak, obtained by carboxymethyl cellulose chromatography of globin prepared by acid acetone precipitation of a whole cell lysate. In order to calculate the globin chain synthetic ratio radioactivity has been corrected for the number of valine present in each chain. There is no significant difference in the δβ-chain synthetized by the 2 brothers. The actual synthesis of δβ-chain is given by the radioactivity present in the peak which is low (table II) The low amount of δβ-chain synthesized may reflect the known

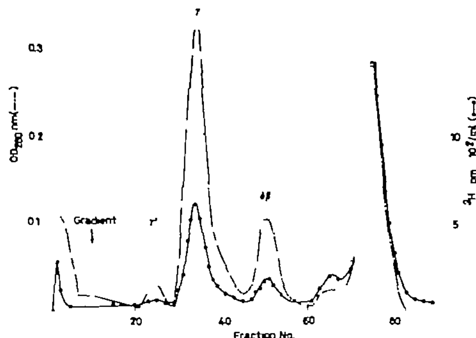


Fig. 2. Globin synthesis in hymozygous haemoglobin Lepore (L. P.). Carboxymethyl cellulose column chromatography of 20 mg of globin, labelled in the reticulocytes with ^{3}H-L-valine, as reported in Technical Procedures.

Table II. Globin chain synthesis by reticulocytes from patients homozygous Hb Lepore

Subject	cpm incorporated				Ratio	
		γ	γ'	δβ	α/(γ + γ')	(δβ + γ + γ')
L P	9,828	4,020	468	932	2.19	1.80
P P	88,918	23,694	5,114	5,571	3.08	2.58

phenomenon that the synthesis of δβ-chain, like that of δ-chain, stops early during erythroid maturation, so that it has almost ceased when the red blood cells are released into the peripheral blood [6, 10]. From table II it is also apparent that the ratio α/(γ+γ') (or α/(δβ+γ+γ')) differs from unity. The degree of imbalance, i.e. the excess of α-chain synthesized, is different in the 2 brothers. Namely it is higher in patient P P who presents more serious clinical manifestations.

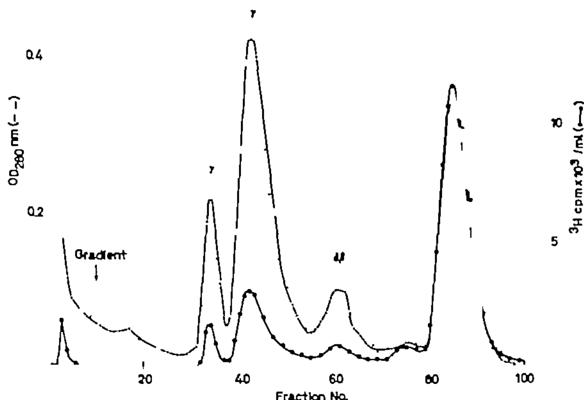


Fig. 3. Globin synthesis in homozygous haemoglobin Lepore (P P.). Carboxymethyl cellulose column chromatography of 20 mg of globin, labelled in the intact reticulocytes with ^3H *L*-valine, as reported in Technical Procedures.

Discussion

This is the first instance where globin chain synthesis has been investigated in patients homozygous for Hb Lepore. Although they are genetically identical for the $\delta\beta$-allele, the severity of disease, such as the clinical manifestations typical of thalassaemia and the degree of anaemia, is very different. The older showed a mild haemolytic anaemia. Haemoglobin was only slightly below normal. He did not need transfusions and showed the clinical features of thalassaemia intermedia. The younger brother showed a haemoglobin concentration very much below normal values and in fact he was regularly transfused. The fact that in this patient the reticulocyte count was not so high as expected by the degree of anaemia could be explained by the inhibition of the red cell production by the bone marrow probably due to the regular transfusions or by the more severe ineffective erythropoiesis this in turn could explain the lower bilirubin level found in this subject in respect to the older brother

We have noticed a correlation between the milder disease and the higher rate of production of γ-chain to compensate the excess of α-chain. This mechanism of γ-chain activation (or γ-chain locus more active) may have been inherited differently by the 2 brothers. Genes other than that involved in Hb synthesis and the environmental factors

may have a role in the severity of the disease. Since in the 2 patients the only Hb present, beside Hb Lepore, is Hb F one possible explanation for the different severity of the disease would be that the red cells contain different amounts of Hb F and consequently have a different life span, depending on the amount of Hb F as in β-thalassaemia [11] and that the 2 brothers contain different amounts of the two populations of cells.

We conclude that our results confirm the idea that the severity of the clinical manifestations in Hb Lepore depends, as in β-thalassaemia, on the degree of globin chain imbalance.

References

1 Baglioni, C. The fusion of two peptide chains in hemoglobin Lepore and its interpretation as a genetic deletion. Proc. natn Acad. Sci. USA 48 1880–1886 (1962).

2 Baglioni, C. and Ventruto, V Human abnormal hemoglobins. II. A chemical study of hemoglobin Lepore from a homozygote individual. Eur J Biochem. 5 29–32 (1968).

3 Barnabas, J and Müller C. J Hemoglobin Lepore (Hollandia). Nature, Lond. 194 931–932 (1962).

4 Burka, E. R. and Marks, P A. Protein synthesis in erythroid cells. II. Polyribosome function in intact reticulocytes. J molec. Biol. 9 439–451 (1964).

5 Clegg, J B Naughton, M. A., and Weatherall, D. J Abnormal human haemoglobins. Separation and characterization of the and β chains by chromatography and the determination of two new variants, Hb Chesapeak and Hb J (Bangkok). J molec. Biol. 19 91 108 (1966)

6 Lang, A., White, J M., and Lehmann, H. Synthesis of Hb Lepore (as $\delta\beta$): influence of δ and β nucleotide sequence on synthesis of $\delta\beta$ chain. Nature new Biol. 240 268–271 (1972).

7 Ostertag, W and Smith, E. W Hemoglobin-Lepore-Baltimore, third type of δ,β crossover (δ^{50} β^{86}). Eur J Biochem. 10 371 376 (1969).

8 Quattrin, N. and Ventruto, V Hemoglobin Lepore: its significance for thalassemia and clinical manifestations. Ann. N Y Acad. Sci. 232. 65–73 (1974).

9 Weatherall, D. J The thalassaemia syndromes (Blackwell, Oxford 1955).

10 Weatherall, D J Clegg, J B.; Roberts, A. V and Knox-MacAulay H. H. M. The clinical and chemical heterogeneity of the β-thalassemias. Ann. N. Y Acad. Sci. 232. 83–105 (1974).

11 White, J M Lang, A. Lorkin, P A., Lehmann, H., and Reeve J Synthesis of haemoglobin Lepore. Nature new Biol. 235 208–210 (1972).

Received. December 17 1977
Accepted. October 18, 1978

Benedetta Lappis, 3rd Pediatric Clinic,
University Hospital 'L. Sacco'
Via G. B. Grassi, 74,
20157–Milan (Italy)

Acta haemat. 61 222-225 (1979)

Spectrophotometric Method Applicable to *in vitro* Studies of Heinz Body Formation in Erythrocytes

M Summerfield and G R Tudhope

Department of Pharmacology and Therapeutics, University of Dundee, Ninewells Hospital, Dundee

Key Words. Crystal violet Haemoglobin oxidation Heinz bodies, Spectrophotometry

Abstract. A simple quantitative procedure is described for the estimation of Heinz body formation in erythrocytes, based on the spectrophotometric measurement of the amount of crystal violet absorbed by precipitated Heinz bodies. Good correlation is shown between this method and the counting of Heinz body-containing erythrocytes in a microscopical preparation. This technique is particularly applicable to *in vitro* studies of the comparative effects produced in the erythrocyte by oxidant agents.

Introduction

The formation of Heinz bodies within erythrocytes results from the oxidative degradation of haemoglobin [3 5] Estimation of Heinz bodies has been used as a measure of the effect of oxidant drugs on intraerythrocytic haemoglobin [1 4 9 15] and in studies of erythrocytic changes leading to haemolysis [6 12]

Various stains have been used to demonstrate Heinz bodies in erythrocyte preparations and to allow counting of the number of cells containing Heinz bodies or of the number of such bodies per cell. Other workers have measured Heinz body formation by weighing the precipitate formed after lysis of the erythrocytes [5] or by the use of a radioactive label [7] In this report, a simple quantitative procedure for the estimation of Heinz body formation is described, based on spectrophotometric measurement of the amount of stain absorbed by Heinz bodies precipitated from a lysed erythrocyte preparation.

Methods

Venous blood from healthy subjects was collected in tubes containing lithium heparin. After centrifugation, the plasma and leucocyte layers were removed. The erythrocytes were washed three times with 0.15 *M* NaCl, and then diluted with buffered saline (0.15 *M* NaCl, 0.01 *M* phosphate buffer pH 7.5) so that the haemoglobin concentration of the resulting cell suspension was 4 g/dl.

A portion of this erythrocyte suspension was incubated with phenylhydrazine (10^{-4} *M*) for

15 min at 37 °C; previous experiments had shown that this procedure resulted in 100% of the erythrocytes containing Heinz bodies. The cells were then washed twice with buffered saline to remove excess phenylhydrazine and made up to the original volume. Suitable volumes of the phenylhydrazine-treated cell suspension and of the untreated cell suspension were then mixed so as to give series of erythrocyte suspensions, each of 2 ml, calculated to contain range of percentages of Heinz body-containing cells from 0 to 100%. From each mixed cell suspension, a sample of cells was stained with crystal violet [14], smears were made and the percentage of cells containing Heinz bodies were counted. The remainder of the mixed cell suspensions were centrifuged and the packed erythrocytes were lysed by the addition of 8 ml distilled water. After 5 min the tubes were centrifuged at 2,500 *g* for 10 min and the pellets were washed once with distilled water and once with 0.15 *M* NaCl. Each of the small dark pellets were then dispersed in 2 ml crystal violet CI 42555 (BDH) solution; this solution had an absorbance of 0.9 at 580 nm in glass cells with 2 mm light path and was prepared by dissolving 0.25 g of stain in 1 litre of 0.15 *M* NaCl. A control tube containing stain solution only was also prepared. After 1 h at 30 °C the tubes were centrifuged at 2,500 *g* for 10 min and the absorbance of the supernatants determined on a Unicam SP500 spectrophotometer at 580 nm in glass cells with 2 mm light path or by diluting 1:5 and using cells with 10 mm light path. The difference in absorbance between each supernatant and the control tube containing stain alone was determined.

Results

The difference in absorbance between the control stain solution and the supernatant after centrifugation was a measure of the amount of stain absorbed by the Heinz bodies. There was a close linear relationship between the difference in absorbance and the percentage of cells containing Heinz bodies calculated to be present in each mixed erythrocyte suspension (fig. 1). The high concentration of stain used was required to ensure linearity of results.

The smears made from each erythrocyte mixture were examined by two observers who were unaware of the calculated percentage of cells containing Heinz bodies in each

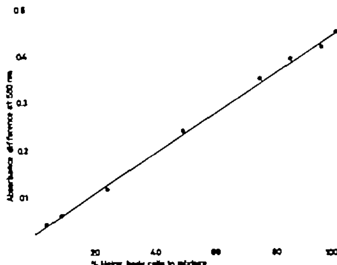


Fig. 1. The relationship between change in absorbance at 580 nm due to uptake of crystal violet by Heinz body preparations, and the percentage of Heinz body-containing cells calculated to be present in the mixtures.

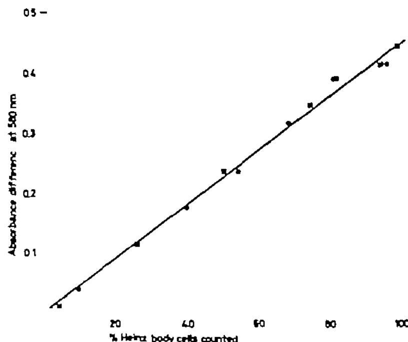


Fig. 2. The relationship between change in absorbance at 580 nm due to uptake of crystal violet by Heinz body preparations, and the percentage of Heinz body-containing cells in the mixtures, counted by two observers (● ■).

sample. There was close agreement between the two counts of Heinz body-containing cells (fig 2) The difference between the percentages of cells with Heinz bodies as counted from the smears and as calculated from the mixture of erythrocyte suspensions was, in each case, less than 10%, and there was a close linear relationship between the Heinz body counts and the difference in absorbance of stain solution measured (fig. 2).

Discussion

Most studies of Heinz bodies in erythrocytes have depended upon the microscopical examination of stained smears, the number of cells containing Heinz bodies or the number of bodies per cell being counted. Several dyes have been used including crystal violet [1 4] methyl violet [3] brilliant green [11] rhodanile blue [13] brilliant cresyl blue [6] nile blue sulphate [10] and new methylene blue [8] *Cruz* [2] haemolyzed blood with distilled water and used the turbidity of the resulting haemolysate as a measure of Heinz body formation. To obtain a more quantitative estimate, *Jandl et al* [5] weighed the precipitate obtained from the lysis of erythrocytes containing Heinz bodies and *Martinez et al* [7] labelled erythrocytes with radiochromium and measured the radioactivity of the precipitate after lysis. The method described in the present report is a simple spectrophotometric technique which gives comparative quantitative values in good agreement with the results of microscopical examination of smears and with the known percentages of Heinz body-containing cells in artificially prepared mixtures of erythrocytes.

This spectrophotometric method is applicable to *in vitro* studies of factors causing the oxidation of haemoglobin in erythrocytes and of the early erythrocytic prehaemolytic changes. It is not of value for the identification of relatively small numbers of cells containing Heinz bodies occurring *in*

vivo in clinical situations and clearly it does not allow direct determination of the size and number of Heinz bodies. It provides a measure of the total amount of Heinz body formation in the erythrocyte sample. This method is particularly of value in studies *in vitro* of the actions on erythrocytes of oxidant drugs, as it allows a direct comparison to be made of the effects of different drugs, of different concentrations of a drug or of differing conditions of incubation.

Acknowledgements

The technical assistance of Mrs. *E. McQueen* is gratefully acknowledged. This research programme was supported by a grant from the Scottish Hospital Endowments Research Trust.

References

1 Beutler, E., Dern, R. J. and Alving, A. S.: The hemolytic effect of primaquine. VI. An *in vitro* test for sensitivity of erythrocytes to primaquine. J. Lab. clin. Med. *45*: 40–50 (1955).

2 Cruz, W. O.: Acetylphenylhydrazine anemia. I. The mechanism of erythrocyte destruction and regeneration. Am. J. med. Sci. *202*: 781–798 (1941).

3 Harley, J. D. and Mauer, A. M.: Studies on the formation of Heinz bodies. II. The nature and significance of Heinz bodies. Blood *17*: 418–433 (1961).

4 Hopkins, J. and Tudhope, G. R.: The effects of drugs on erythrocytes *in vitro*: Heinz body formation, glutathione peroxidase inhibition and changes in mechanical fragility. Br. J. clin. Pharmacol. *1*: 191–195 (1974).

5 Jandl, J. H., Engle, L. K., and Allen, D. W.: Oxidative hemolysis and precipitation of hemoglobin. I. Heinz body anemias as an acceleration of red cell aging. J. clin. Invest. *39*: 1818–1836 (1960).

6 Lubin, A. and Desforges, J. F.: Effect of Heinz bodies on red cell deformability. Blood *39*: 658–665 (1972).

7 Martinez, M., Lo, A., Cohen, G., and Kaplan, H.: Radioactive (chromium-51) Heinz bodies. Nature, Lond. *210*: 1064–1066 (1966).

8 Menzel, D. B., Slaughter, R. J.; Bryant, A. M., and Jauregui, H. O.: Heinz bodies formed in erythrocytes by fatty acid ozonides and ozone. Archs envir. Hlth *30*: 296–301 (1975).

9 Miller, A. and Smith, H. C.: The intracellular and membrane effects of oxidant agents on normal red cells. Br. J. Haemat. *19*: 417–428 (1970).

10 Schröter, W. and Tillman, W.: Heinz body susceptibility of red cells and exchange transfusion. Acta haemat. *49*: 74–79 (1973).

11 Schwab, M. L. L. and Lewis, A. E.: An improved stain for Heinz bodies. Am. J. clin. Path. *51*: 673–675 (1969).

12 Sears, D. A., Friedman, J. M., and White, D. R.: Binding of intracellular protein to the erythrocyte membrane during incubation: the production of Heinz bodies. J. Lab. clin. Med. *86*: 722–732 (1975).

13 Simpson, C. F.; Carlisle, J. W. and Mallard, L.: Rhodanile blue; rapid and selective stain for Heinz bodies. Stain Technol. *45*: 221–223 (1970).

14 Tudhope, G. R. and Hopkins, J.: Lipid peroxidation in erythrocytes. Acta haemat. *51*: 29–39 (1974).

15 Tudhope, G. R. and Leece, S. P.: Red-cell catalase and the production of methaemoglobin, Heinz bodies and changes in osmotic fragility due to drugs. Acta haemat. *45*: 290–302 (1971).

Received, July 5, 1978
Accepted, September 22, 1978

Dr. G. R. Tudhope,
Department of Therapeutics,
Ninewells Hospital,
Dundee DD2 1UD (Scotland)

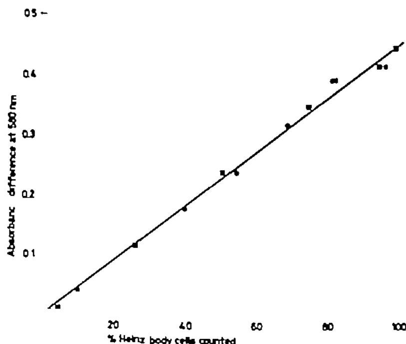


Fig. 2. The relationship between change in absorbance at 580 nm due to uptake of crystal violet by Heinz body preparations, and the percentage of Heinz body-containing cells in the mixtures, counted by two observers (● ■).

sample. There was close agreement between the two counts of Heinz body-containing cells (fig. 2) The difference between the percentages of cells with Heinz bodies as counted from the smears and as calculated from the mixture of erythrocyte suspensions was, in each case, less than 10%, and there was a close linear relationship between the Heinz body counts and the difference in absorbance of stain solution measured (fig 2)

Discussion

Most studies of Heinz bodies in erythrocytes have depended upon the microscopical examination of stained smears, the number of cells containing Heinz bodies or the number of bodies per cell being counted. Several dyes have been used including crystal violet [1 4] methyl violet [3] brilliant green [11] rhodanile blue [13] brilliant cresyl blue [6] nile blue sulphate [10] and new methylene blue [8] *Cruz* [2] haemolyzed blood with distilled water and used the turbidity of the resulting haemolysate as a measure of Heinz body formation. To obtain a more quantitative estimate, *Jandl et al* [5] weighed the precipitate obtained from the lysis of erythrocytes containing Heinz bodies and *Martinez et al* [7] labelled erythrocytes with radiochromium and measured the radioactivity of the precipitate after lysis. The method described in the present report is a simple *spectrophotometric technique* which gives comparative quantitative values in good agreement with the results of microscopical examination of smears and with the known percentages of Heinz body-containing cells in artificially prepared mixtures of erythrocytes.

This spectrophotometric method is applicable to *in vitro* studies of factors causing the oxidation of haemoglobin in erythrocytes and of the early erythrocytic prehaemolytic changes. It is not of value for the identification of relatively small numbers of cells containing Heinz bodies occurring *in*

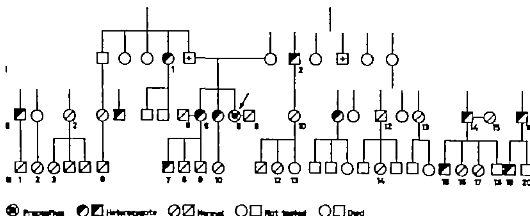


Fig. 1. Family pedigree of the proposita.

family to be affected with the classical δ-β-thalassaemia minor haematological picture (microcytosis, pseudopolyglobulia with or without anaemia, normal or low haemoglobin A_2 and high haemoglobin F concentration with variable amount of RBC with haemoglobin F).

Figure 1 shows the complete pedigree of the family containing the proposita (II-8), 3 'uncertain' babies (III-13, III-18 and III 20), 3 married couples (II-5, II-9 and II-15) and 12 affected and 19 normal members.

Discussion

Our patient showed a normocytic anaemia with a dysmorphia, clear regenerative traits, erythrocytic hyperresistance *in vitro*, hypersideraemia, and no evidence of catabolic products of the haemoglobin in her blood or urine. Most important for the diagnosis of the disease was the acid elution test which showed 100% of RBC to contain haemoglobin F

More accurate methods showed the total absence of haemoglobin A_1 and A_2, and the abolition of synthesis of δ- and β-chains with alterated α-γ-globin synthesis ratio. After almost 2 years the patient remains stabilized.

All the 9 known homozygous patients from the literature (including ours) presented sufficient clinical and haematological data to be diagnosed. 6 cases were in apparent good health and the firstly described patient was seriously anaemic and ill at the time of diagnosis. 2 other healthy cases suffered once from a serious anaemic crisis. The diagnosis was made during childhood on 5 occasions. *Only in our case was it made* above 31 years of age. All the patients had hepatomegaly or splenomegaly or both, and only 5 had a variable degree of radiological changes in the bones. Table I shows the comparative available data of the 9 cases. It is interesting to note that the MCV of the 5 adults reported was 83.2 ± 3.3 fl, without microcytic change. In 3 cases in which reticulocyte counts and serum iron were expressed (cases 5 8 and 9) both parameters were over the normal values.

Homozygous δ-β-thalassaemia is a rare and benign form of thalassaemia major. Including ours, only 9 cases have been described so far Until now the disease seems to have been confined to the Mediterranean area. Since the interesting cooperative ana-

Acta haemat. 61 226–229 (1979)

Homozygous Delta-Beta-Thalassaemia in a Spanish Woman

E. Gimferrer, M. Baiget and M. L. Rutllant[1]

Servicio de Hematología, Hospital de la Santa Creu i Sant Pau, Barcelona

Key Words. Delta-beta-thalassaemia · Thalassaemia

Abstract. Haematological and clinical data from a Spanish anaemic woman with 100% haemoglobin F are reported. The results together with the family studies confirmed this to be a new case of homozygous δ-β-thalassaemia.

Introduction

δ-β-Thalassaemia has been largely described in its heterozygous form, in double heterozygous association with β-thalassaemia, and less frequently in double heterozygous association with structural haemoglobin variants. The homozygous form of this syndrome has been reported in a small number of cases. The first case was described by *Brancati and Baglioni* [1] in an Italian patient. Thereafter the syndrome was also found in 3 new Italian patients [4], 3 Arabic patients [3] and a Greek patient [5]. Our paper deals with a further case in a Spanish woman, with haematological, biochemical and clinical studies and with a family study of 37 members.

[1] With the technical collaboration of *E. del Rio* and *N. Artigas*.

Case Report

A white 55-year-old female, from the province of Valencia, Spain, married, without children, entered the Hospital de la Santa Creu i Sant Pau of Barcelona for operation of an inguinal hernia. Her general clinical condition was good. The clinical study showed only a moderate pallor of the skin, and a splenomegaly of 6 cm. The haematological data are given in table I. The cellulose acetate and agar-gel electrophoresis, the scanning and the DEAE-cellulose chromatography of the haemolysate all showed the typical pattern of haemoglobin F.

The isopropanol stability test and the thermal resistance of the haemoglobin were both normal. The study of globin chain synthesis showed an alpha/gamma ratio of 1.24 (specific radioactivity). Observation of the bone marrow aspirate of the patient showed an erythropoietic hyperplasia which amounted to 70% of the total cellularity with 80% of sideroblasts and increased reticular iron stores. The radiology of the skull was normal.

The screening of 37 relatives of the patient showed several members of both branches of the

liamson, R., Russo, G., Musumeci, S. Schilliro, G. Tsistrakis, G. A., Charache, S.; Wood, W. G. Clegg, J. B., and Weatherall, D. J. Delta beta thalassaemia is due to a gene deletion. Cell 9 71–80 (1976).

3 Ramot, B., Ben-Bassat, I., Gafni, D. and Zaanoon, R. A family with three beta-delta thalassaemia homozygotes. Blood 35 158–165 (1970).

4 Silvestroni, E., Bianco, I., and Reitano, G. Three cases of homozygous beta-delta thalassaemia (or microcythaemia) with high haemoglobin F in Sicilian family. Acta haemat. 40. 220–229 (1968).

5 Tsistrakis, G. A., Amarantos, S. P. and Koutouris, L. L.: Homozygous beta-delta thalassaemia. Description of a case and review of the literature. Acta haemat. 51 185–191 (1974).

Received: July 26, 1978
Accepted: September 28, 1978

Dr Enric Gimferrer
Servicio de Hematología,
Hospital de la Santa Creu i Sant Pau,
Barcelona, 25 (Spain)

Table I. Homozygous δ-β-thalassaemia common haematological data of the 9 cases described

	Origin, reference and year								
	Italian [1] 1966	Italian [4] 1968			Arabic [3] 1970			Greek [5] 1974	Spanish [this study] 1978
Case No.	1	2	3	4	5	6	7	8	9
Age	31	5	3	1.25	25	12	9	22	55
Sex	F	F	F	F	M	M	F	M	F
Hb, g/dl		12.7	10	6	12.3			4.2	10.7
PCV	31	43	30	20	42			16	32
RBC, $\times 10^{12}$/l	3.9	5.3	4.2	3.0	4.9			1.8	3.98
Reticulocyte count, $\times 10^9$/l					171			144	183
MCV fl	80	82	71	66	85			88	81
MCHC, g/dl		29.5	33.3	30	34			26.2	33.6
Osmotic fragility	decr	decr	decr	decr	decr			decr	decr
Serum iron, μmol/l					29.9			28.1	28.3
RBC with Hb F %	100	100	100	100	100		100		100
Hb F %	100	100	100	100	100	100	100	100	100
Hb A, %	0	0	0	0	0	0	0	0	0
Hb A_2, %	0	0	0	0	0	0	0	0	0
RBC dysmorphia	AHKQDB	I	IB	IB	HID			AHIKQD	AIKDV

F = Female M = male A = anisocytosis H = hypochromia I = microcytosis K = poikilocytosis D = target cells B = erythroblastaemia V = ovalocytosis.

lytical and hybridization studies carried out by *Ottolenghi et al.* [2] it seems that the disease, as well as hereditary persistence of fetal haemoglobin (HPFH) is due to a DNA deletion at the level of γ-δ-β cluster with the suppression of the production of δ- and β RNA and the respective globin chains. The haematological and clinical picture which differentiates homozygous δ-β-thalassaemia from homozygous HPFH seems to be dependent on some unknown characteristics of the deletion in each one of the diseases. Thus, in the former with more critical deletion the γ-RNA and γ-globin synthesis would not be as spared as in the latter and this would lead to an unbalanced production of chains in the erythroid precursors and to intra- and extramedullary haemolysis, anaemia, heterogeneous F haemoglobin distribution in the erythrocytes, and to the different clinical patterns of the disease.

References

1 Brancati, C. and Baglioni, C.: Homozygous beta-delta thalassaemia (beta-delta microcythaemia). Nature, Lond. 212 262–264 (1966)

2 Ottolenghi, S., Comi, P., Giglioni, B., Tolstoshev P Lanyon, W G.; Mitchell, G J., Wil

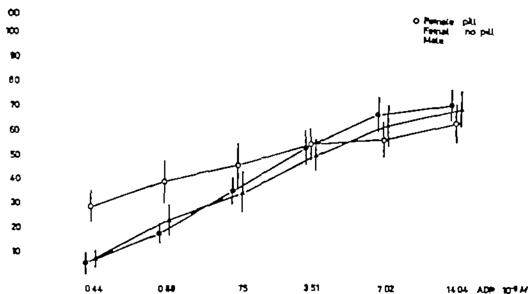


Fig. 1. Peak of aggregation (mean ± SD)

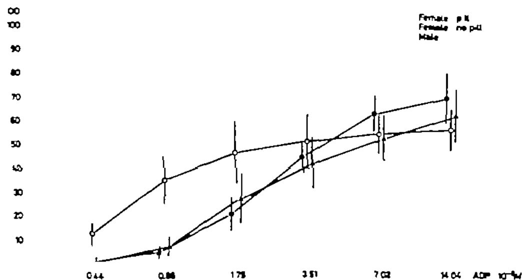


Fig. 2. Disaggregation (mean ± SD).

Acta haemat. 61 230–232 (1979)

Human Platelet Aggregation Curve and Oral Contraception

C M D Montanari A Vittoria U Rossi and P Sala

Istituto di Semeiotica Medica, Università degli Studi di Siena, Siena, and Laboratorio di Ematologia e Centro Trasfusionale, Ospedale Civile di Legnano, Milano

Key Words. Oral contraceptives Human platelets ADP

Abstract. In a series of *in vitro* experiments, the authors investigated the different aspects of the aggregation curves of human blood platelets taken from a group of women who received oral contraceptives, another group of women who did not receive oral contraceptives and a group of men. The platelets were subjected to ADP stimulation. The authors showed that at the lower doses (0 44 and 0.88 10^{-6} *M*) of ADP the aggregation and the disaggregation of the platelets from the women who received oral contraceptives was significantly greater than that of the two other groups. The authors proposed that these may be due to an increase of the sensitivity of the platelets to the aggregating stimuli in that group of women who were under oral contraception.

The influence of the oral contraceptives on the physiology of blood coagulation factors has been investigated since their first appearance. The oestrogen-based contraceptives showed the capacity to enhance platelet sensitivity to the aggregating stimuli [*Clayman et al.* 1973 *Hedlin*, 1975]

We examined the profile of the platelet response in women under oral contraception, compared with women not under oral contraception and with men.

28 normal subjects (blood donors and students) were selected for this trial, 8 women (median age 24 years, range 21–28) in oral contraception at least for 2 months, 6 women not under oral contraception (median age 30 years, range 23–39) and 14 men (median age 32 years, range 21–45). They were screened for their recent history of medication to avoid those cases whose concomitant medications could affect platelet response. The details of the method are described in a previous work [*Sacchetti et al.*, 1973].

Results and Conclusions

Three aspects of the aggregation curve were considered, the peak, the speed and the maximum of disaggregation. As far as the peak of aggregation is concerned, women under oral contraception showed a higher sensitivity to the lower doses of ADP than the other two groups (fig. 1). The be-

Acta haemat. 61 233-234 (1979)

Circulating Anticoagulant in Patients with Gaucher's Disease?

D Benjamin H Joshua, D Douer M Shaklai Y Krugliac and J Pinkhas

Department of Medicine 'D' and the Clinical Laboratory Beilinson Medical Center
Tel Aviv University Medical School, Petah Tikva

Key Words. Circulating anticoagulants Sphingolipids Gaucher

Abstract. A case of Gaucher's disease is presented, in which prolonged partial thromboplastin time was found. The possibility of circulating sphingolipids responsible for this abnormality is raised.

Patient's with Gaucher's disease often present hemorrhagic phenomena, commonly attributed to thrombocytopenia. We presently report a defect in the coagulation system in a patient with Gaucher s disease, manifesting as prolonged partial thromboplastin time (PTT). The question whether circulating sphingolipids are responsible for this abnormality is discussed.

Case Report

Routine coagulation studies (table 1) were per formed in 66-year-old patient with Gaucher's disease. 20 years ago he underwent uneventful cholecystectomy without hemorrhagic phenomena.

Marked thrombocytopenia, prolonged bleeding time and PTT measured according to the method of *Proctor and Rapaport* [4] were found. The titration of the thromboplastic factors revealed nor mal values for factors VIII and IX and low nor mal values for factor XI, assayed according to the technique of *Rapaport et al.* [5]. when 1 vol of the patient's plasma was added to 9 vol of normal control plasma, the PTT of the latter was found to be prolonged. On the other hand, when 1 vol of normal control plasma was added to 9 vol of the patient's plasma, no correction of the prolonged PTT was achieved (table 1). W compared the effect on PTT of celite-absorbed (factor XI deficient) patient's plasma to plasma sample obtained from patient with factor XI deficiency When 1/10 isotonic veronal buffer-diluted normal plasma was used as substrate and mixed with celite-absorbed patient's plasma, the PTT was found to be prolonged (39 min 6 sec) as compared to the result obtained by mixing the substrate with factor XI deficient control plasma (35 min 6 sec). Similar results were obtained when factor XI-deficient plasma was used as a substrate in testing the *correcting ability of either normal (93 sec) or patient's* celite-absorbed plasma (125 sec). In all the experiments the prolongation of normal PTT by the patient's plasma was evident and found in three consecutive experiments.

Discussion

In addition to thrombocytopenia, the presently reported patient showed a prolonged PTT and low borderline factor XI activity *Bolkan and Sawitsky* [1] found that in their 11 patients with Gaucher's disease a slightly prolonged PTT was found and low levels of factors VII VIII, X and especially factor IX were found in some of these pa-

tween-group difference disappeared above 0 88 10^{-4} *M* ADP The groups were different at the lower doses of ADP (below 1 75 10^{-4} *M*), concerning disaggregation as well (fig. 2) The groups were not statistically different regarding the speed of aggregation.

These preliminary findings suggest that the platelet threshold is lowered by the presence of oestrogenic contraceptives, with a probable influence on the hemostatic process in a more complex fashion [*Elkeles et al.* 1968 *Davies et al* 1976]

References

Clayman R. Gadd, R. E. A., and Heber D The *in vitro* effects of several progestogens, estrogens and non-steroidal compounds with estrogenic activity on adenosine diphosphate induced guinea-pig platelet aggregation. Experientia 29 95 (1973).

Davies, T Fieldhams, G and McNicol, G P., The effects of therapy with oestriol succinate and ethinyl oestradiol on the emostatic mechanism in post menopausal women. Thromb. Haemost. 35 403 (1976).

Elkeles, R. S., Hampton, J R., and Mitchell, J R. A. Effect of oestrogens on human platelet behaviour Lancet *II.* 315 (1968).

Hedlin, A. M., The effect of oral contraceptive estrogen on blood coagulation and fibrinolysis. Thromb. Diath. haemorrh. 33 370 (1975).

Sacchetti, G., Bellani, D., Montanari, C., and Gibelli, A.. Effects *in vitro* of some cardiovascular drugs and other agents on human platelet aggregation. Thromb Diath. haemorrh. 29 190 (1973).

Received. June 26, 1978
Accepted. October 5 1978

C. M. D Montanari,
Istituto di Semeiotica Medica,
Università degli Studi di Siena, Siena (Italy)

41: 233–234 (1979)

_ Anticoagulant in Patients with Gaucher's Disease?

H Joshua D Douer M Shaklai Y Kroglac and J Pinkhas

of Medicine 'D' and the Clinical Laboratory Beilinson Medical Center,
University Medical School, Petah Tikva

_ anticoagulants Sphingolipids Gaucher

A case of Gaucher's disease is presented, in which prolonged partial thrombo- time was found. The possibility of circulating sphingolipids responsible for this 'y is raised.

..ts with Gaucher's disease often hemorrhagic phenomena, commonly ..ted to thrombocytopenia. We pres report a defect in the coagulation sys- in a patient with Gaucher's disease ..ting as prolonged partial thrombo- .. time (PTT). The question whether ..ting sphingolipids are responsible for abnormality is discussed.

Case Report

..tion coagulation studies (table 1) were per ..med in a 66-year-old patient with Gaucher's ..ease. 20 years ago he underwent uneventful splenectomy without hemorrhagic phenomena.

Marked thrombocytopenia, prolonged bleeding time and PTT measured according to the method of Proctor and Rapaport [4] were found. The assays of the thromboplastic factors revealed normal values for factors VIII and IX and low normal values for factor XI, assayed according to the technique of Rapaport et al. [5]: when 1 vol of the patient's plasma was added to 9 vol of normal control plasma, the PTT of the latter was found to be prolonged. On the other hand, when 1 vol of normal control plasma was added to 9 vol of the patient's plasma, no correction of the prolonged PTT was achieved (table 1). We compared the effect on PTT of celite-absorbed (factor XI deficient) patient's plasma to plasma sample obtained from patient with factor XI deficiency. When 1:10 normal veronal buffer-diluted normal plasma was used as substrate and mixed with celite-absorbed patient's plasma, the PTT was found to be prolonged (39 min 6 sec) as compared to the result obtained by mixing the substrate with factor XI deficient control plasma (35 min 6 sec). Similar results were obtained when factor XI-deficient plasma was used as a substrate in testing the correcting ability of either normal (91 sec) or patient's celite-absorbed plasma (125 sec). In all the experiments the prolongation of normal PTT by the patient's plasma was evident and found in three consecutive experiments.

Discussion

In addition to thrombocytopenia, the presently reported patient showed a prolonged PTT and low borderline factor XI activity. Boklan and Sawitsky [1] found that in their 11 patients with Gaucher's disease a slightly prolonged PTT was found and low levels of factors VII, VIII, X and especially factor IX were found in some of these pa-

tween-group difference disappeared above 0 88 10^{-4} *M* ADP The groups were different at the lower doses of ADP (below 1 75 10^{-4} *M*), concerning disaggregation as well (fig. 2) The groups were not statistically different regarding the speed of aggregation.

These preliminary findings suggest that the platelet threshold is lowered by the presence of oestrogenic contraceptives, with a probable influence on the hemostatic process in a more complex fashion [*Elkeles et al* 1968 *Davies et al* 1976]

References

Clayman, R., Gadd, R. E. A., and Heber D The *in vitro* effects of several progestogens, estrogens and non-steroidal compounds with estrogenic activity on adenosine diphosphate induced guinea-pig platelet aggregation. Experientia 29 95 (1973).

Davies, T., Fieldhause, G and McNicol, G P. The effects of therapy with oestriol succinate and ethinyl oestradiol on the emostatic mechanism in post-menopausal women. Thromb. Haemost. *35* 403 (1976).

Elkeles, R. S., Hampton, J R., and Mitchell, J R. A.. Effect of oestrogens on human platelet behaviour Lancet *ii*. 315 (1968).

Hedlin, A. M. The effect of oral contraceptive estrogen on blood coagulation and fibrinolysis. Thromb Diath. haemorrh. *33* 370 (1975)

Sacchetti, G Bellani, D Montanari, C., and Gibelli, A.. Effects *in vitro* of some cardiovascular drugs and other agents on human platelet aggregation. Thromb. Diath. haemorrh. 29 190 (1973).

Received. June 26, 1978
Accepted. October 5, 1978

C. M. D Montanari,
Istituto di Semeiotica Medica,
Università degli Studi di Siena, Siena (Italy)

Letter to the Editor

Acta haemat. *61* 235 (1979)

Heterozygous Beta Thalassaemia and Cold Agglutinin Disease in a Surinamic Man

M. E. L. v. d. Burg and H. Mulder

Department of Internal Medicine, Municipal Hospital, Bergweg, Rotterdam

As far as we can determine this is the first report of this nature.

Case Report

A 48-year-old Surinamic man arrived in the Netherlands 2 months before hospitalization. The descriptive diagnosis, haemolytic anaemia, had been made 3 years previously in Surinam, for which he received 30 mg prednisone daily. H had complaints of palpitation, dyspnoea, tingling in the hands, increasing fatigue and cramps in the extremities without Raynaud's phenomenon. On physical examination of this ill looking man we noted Cushingoid face, yellow sclerae, no lymphadenopathy or hepatosplenomegaly.

Laboratory Findings

ESR 110 mm, Hb 6.1 g/dl, Ht 0.19, reticulocytes 4%, WBC 6.0×10^9/l, the peripheral blood picture under normal circumstances presents aggregation of the erythrocytes and normal picture of the erythrocytes after warming the slide. Plasma iron 36 μmol/l, total plasma iron binding capacity 37 μmol/l, total bilirubin 50 μmol/l, indirect fraction 47 μmol/l, LDH 443 U/l, haptoglobin 0 mg/dl. In the bone marrow aspirate the erythropoiesis was three times as active as the myelopoiesis. Red cell survival (^{51}Cr T_{50}) was 18.2 days during prednisone and 15.5 days without prednisone (normal 26–32 days), the heart-spleen-liver index was normal. It was concluded that the patient was indeed suffering from haemolytic anaemia. T determine the nature of this, the following investigations were performed. Hb electrophoretic pattern, HbA_2 4.4% (1.9–3.0%), HbF 1.3% (n 0.5–1.5%). Direct antiglobulin test: negative with anti-IgA, C, M, D, E, positive with anti-complement antibodies. Identification of the antibodies: cold agglutinins and haemolysins of specificity anti-I.

The titre of the cold agglutinin was more than 1/500. The concentration of complement component C_3 was decreased. Immunoelectrophoretic pattern: IgA 100 IU/ml (n 40–225), IgM 150 IU/ml (45–335), IgG 60 IU/ml (n 65–200). IgM consisted f linear band, as seen with paraprotein. Immunofluorescence pattern of the bone marrow: IgA 22%, IgM 24%, IgG 53%, kappa 70% lambda 30% (normal). Reaction of Wassermann, VDRL and Paul Bunnell were negative. Erythrocyte enzyme patterns normal. Vitamin B_{12} concentration 315 pg/ml (normal).

On the basis of these findings the diagnosis of heterozygous β-thalassaemia combined with cold agglutinin disease was established. W slowly diminished and finally stopped prednisone without precipitating any acceleration of the anaemia. The patient returned to Suriname because he felt the cold Dutch weather was the cause of his troubles.

Comment

This patient is suffering from β-heterozygous thalassaemia and cold agglutinin disease. The two types of anaemia appear to be unrelated.

Received: September 20, 1977
Accepted: October 5, 1978

M. E. L. d. Burg,
Department of Internal Medicine,
Municipal Hospital, Bergweg,
Rotterdam (The Netherlands)

Table I. Coagulation studies

Test	Patient	Normal values
Bleeding time, min	8–15	4–7
Clotting time, min	7	5–8
Platelet count, mm³	30–40 × 10³	150–350 × 10³
PTT sec	49–55	30–45
PT sec	13	12–14
Fibrinogen mg%	218	200–400
Factor VIII %	90	50–150
Factor IX, %	100	50–150
Factor XI %	50	50–150

Table II. PTT of mixtures of patient's and normal plasma

Volumes of plasma		PTT sec	
normal	patient	immediate	after 1 h[1]
10	0	37	39
9	1	43	48
0	10	49	55
1	9	50	54

[1] After 1 h of incubation at 37 C.

tients. Factor XI was measured in 1 patient only and was normal [1]

These authors [1] suggested that the prolongation of PTT might be due to an interference with the action of a certain procoagulant possibly a sphingolipid. As presently shown the patient's plasma brought only a slight prolongation of the PTT of normal plasma. These results could suggest that our patient's plasma contains indeed a factor(s) responsible for the prolongation of PTT of normal plasma.

The biochemical disorder in Gaucher's disease results in excessive accumulation of sphingolipids in the reticuloendothelial system and according to *Dawson and Oh* [2] sometimes also in the plasma. It has already been shown that sphingolipids, particularly cerebrosides inactivate or interfere with the action of procoagulants [3] The mechanism of this anticoagulant activity of sphingolipids is yet unclear according to *Hecht et al.* [3] the inhibition of platelet factor 3 as well as other coagulation factors, may be responsible for this phenomenon. Coagulation abnormalities have been found in sea blue histocytosis, a disorder in which phospholipids, particularly sphingolipids, accumulate in the reticuloendothelial system [1]

The question whether sphingolipids act as anticoagulants and interfere with the procoagulant activity and the prolongation of PTT warrants further investigations.

References

1 Bolkan, B F and Sawitsky A. Factor IX deficiency in Gaucher disease. A *in vitro* phenomenon. Archs intern. Med. *136*. 489–492 (1976).

2 Dawson, G and Oh, J Y.. Blood glucosylceramide levels in Gaucher's disease and its distribution amongst lipoprotein fractions. Clinica chim. Acta *75* 149–153 (1977).

3 Hecht, E. Landburu, R. H and Seegers, W H.. Sphingosine as an inhibitor of prothrombin activation with thromboplastin or with threone. Am. J Physiol. *189* 203–208 (1957).

4 Proctor R. R. and Rapaport, S. I.. The partial thromboplastin time with kaolin, a simple screening test for first stage plasma clotting factor deficiencies. Am. J clin. Path. *36*. 212–19 (1961).

5 Rapaport, S. I. Schiffman, S., and Patch, M. L. A *simple specific one stage assay for plasma* thromboplastin antecedent activity J Lab. clin. Med. *57* 771–776 (1976).

Received. June 27 1978
Accepted. October 8, 1978

Prof. J Pinkhas, Department of Medicine 'D
Tel Aviv University Medical School,
Beilinson Medical Center Petah Tikva (Israel)

Letter to the Editor

Acta haemat. 61: 235 (1979)

Heterozygous Beta Thalassaemia and Cold Agglutinin Disease in a Surinamic Man

M. E. L. v. d. Burg and H. Mulder

Department of Internal Medicine, Municipal Hospital, Bergweg, Rotterdam

As far as we can determine this is the first report of this nature.

Case Report

A 48-year-old Surinamic man arrived in the Netherlands 2 months before hospitalisation. The descriptive diagnosis, haemolytic anaemia, had been made 3 years previously in Suriname, for which he received 30 mg prednisone daily. He had complaints of palpitation, dyspnoea, tingling in the hands, increasing fatigue and cramps in the extremities without Raynaud's phenomenon. On physical examination of this ill looking man we noted: Crablingold face, yellow sclerae, no lymphadenopathy or hepatosplenomegaly.

Laboratory Findings

ESR 110 mm, Hb 6.1 g/dl, Ht 0.19, reticulocytes 4%, WBC 6.0 × 10⁹/l, the peripheral blood picture under normal circumstances presents agglutination of the erythrocytes and a normal picture of the erythrocytes after warming the slide. Plasma iron 36 µmol/l, total plasma iron binding capacity 57 µmol/l, total bilirubin 50 µmol/l, indirect fraction 47 µmol/l, LDH 443 U/l, haptoglobin 0 mg/dl. In the bone marrow aspirate the erythropoiesis was three times as active as the myelopoiesis. Red cell survival (^{51}Cr T_{50}) was 18.2 days during prednisone and 15.5 days without prednisone (normal 26–32 days), the heart-spleen-liver index was normal. It was concluded that the patient was indeed suffering from a haemolytic anaemia. To determine the nature of this, the following investigations were performed: Hb electrophoretic pattern, HbA_2 4.4% (n 1.9–3.0%), HbF 1.3% (n 0.5–1.5%). Direct antiglobulin test: negative with anti-IgA, G, M, D, E, positive with anti-complement antibodies. Identification of the antibodies: cold agglutinins and haemolysins of specificity anti-I.

The titre of the cold agglutinin was more than 1/500. The concentration of complement component C_3 was decreased. Immunoelectrophoretic pattern: IgA 100 IU/ml (n 40–225), IgM 150 IU/ml (n 45–335), IgG 60 IU/ml (n 65–200); IgM consisted of a linear band, as seen with a paraprotein. Immunofluorescence pattern of the bone marrow: IgA 22%, IgM 24%, IgG 33%, kappa 70%, lambda 30% (normal). Reaction of Wassermann, VDRL and Paul Bunnell were negative. Erythrocyte enzyme patterns normal. Vitamin B_{12} concentration 315 pg/ml (normal).

On the basis of these findings the diagnosis of heterozygous β-thalassaemia combined with cold agglutinin disease was established. We slowly diminished and finally stopped prednisone without precipitating any acceleration of the anaemia. The patient returned to Suriname because he felt the cold Dutch weather was the cause of his troubles.

Comment

This patient is suffering from β-heterozygous thalassaemia and cold agglutinin disease. The two types of anaemia appear to be unrelated.

Received: September 20, 1977
Accepted: October 5, 1978

M. E. L. v. d. Burg,
Department of Internal Medicine,
Municipal Hospital, Bergweg,
Rotterdam (The Netherlands)

Table I. Coagulation studies

Test	Patient	Normal values
Bleeding time, min	8–15	4–7
Clotting time, min	7	5–8
Platelet count, mm³	30–40 × 10³	150–350 × 10³
PTT sec	49–55	30–45
PT sec	13	12–14
Fibrinogen, mg	218	200–400
Factor VIII	90	50–150
Factor IX,	100	50–150
Factor XI %	50	50–150

Table II. PTT of mixtures of patient's and normal plasma

Volumes of plasma		PTT sec	
normal	patient	immediate	after 1 h[1]
10	0	37	39
9	1	43	48
0	10	49	55
1	9	50	54

[1] After 1 h of incubation at 37 C.

tients. Factor XI was measured in 1 patient only and was normal [1]

These authors [1] suggested that the prolongation of PTT might be due to an interference with the action of a certain procoagulant, possibly a sphingolipid. As presently shown, the patient's plasma brought only a slight prolongation of the PTT of normal plasma. These results could suggest that our patient's plasma contains indeed a factor(s) responsible for the prolongation of PTT of normal plasma.

The biochemical disorder in Gaucher's disease results in excessive accumulation of sphingolipids in the reticuloendothelial system and according to *Dawson and Oh* [2] sometimes also in the plasma. It has already been shown that sphingolipids, particularly cerebrosides, inactivate or interfere with the action of procoagulants [3] The mechanism of this anticoagulant activity of sphingolipids is yet unclear according to *Hecht et al* [3] the inhibition of platelet factor 3 as well as other coagulation factors, may be responsible for this phenomenon. Coagulation abnormalities have been found in sea blue histocytosis, a disorder in which phospholipids, particularly sphingolipids, accumulate in the reticuloendothelial system [1]

The question whether sphingolipids act as anticoagulants and interfere with the procoagulant activity and the prolongation of PTT warrants further investigations.

References

1 Bolkan, B. F and Sawitsky A.. Factor IX deficiency in Gaucher disease. A *in vitro* phenomenon. Archs intern. Med. *136.* 489–492 (1976).

2 Dawson, G and Oh, J Y.. Blood glucosylceramide levels in Gaucher's disease and its distribution amongst lipoprotein fractions. Clinica chim. Acta *75* 149–153 (1977).

3 Hecht, E. Landburu, R. H., and Seegers, W H.. Sphingosine as an inhibitor of prothrombin activation with thromboplastin or with threone. Am. J Physiol. *189* 203–208 (1957).

4 Proctor R. R. and Rapaport, S. L. The partial thromboplastin time with kaolin, a simple screening test for first stage plasma clotting factor deficiencies. Am. J clin. Path. *36.* 212–219 (1961).

5 Rapaport, S. I. Schiffman, S., and Patch, M. L. A simple specific one stage assay for plasma thromboplastin antecedent activity J Lab. clin. Med. *57* 771–776 (1976).

Received. June 27 1978
Accepted. October 8, 1978

Prof. J Pinkhas, Department of Medicine 'D
Tel Aviv University Medical School,
Beilinson Medical Center Petah Tikva (Israel)

Letter to the Editor

Acta haemat. 61: 235 (1979)

Heterozygous Beta-Thalassaemia and Cold Agglutinin Disease in a Surinamic Man

M. E. L. v. d. Burg and H. Mulder

Department of Internal Medicine, Municipal Hospital, Bergweg, Rotterdam

As far as we can determine this is the first report of this nature.

Case Report

A 43-year-old Surinamic man arrived in the Netherlands 2 months before hospitalisation. The descriptive diagnosis, haemolytic anaemia, had been made 3 years previously in Suriname, for which he received 30 mg prednisone daily. He had complaints of palpitation, dyspnoea, tingling in the hands, increasing fatigue and cramps in the extremities without Raynaud's phenomenon. On physical examination of this ill looking man we noted a Cushingoid face, yellow sclerae, no lymphadenopathy or hepatosplenomegaly.

Laboratory Findings

ESR 110 mm, Hb 6.1 g/dl, Ht 0.19, reticulocytes 4%, WBC 6.0 × 10^9/l, the peripheral blood picture under normal circumstances presents agglutination of the erythrocytes and a normal picture of the erythrocytes after warming the slide. Plasma iron 36 µmol/l, total plasma iron binding capacity 37 µmol/l, total bilirubin 50 µmol/l, indirect fraction 47 µmol/l, LDH 443 U/l, haptoglobin 0 mg/dl. In the bone marrow aspirate the erythropoiesis was three times as active as the myelopoiesis. Red cell survival (^{51}Cr T_{50}) was 18.2 days during prednisone and 15.5 days without prednisone (normal 26–32 days), the heart-spleen-liver index was normal. It was concluded that the patient was indeed suffering from a haemolytic anaemia. To determine the nature of this, the following investigations were performed: Hb electrophoretic pattern, HbA_2 4.4% (n 1.9–3.0%), HbF 1.3% (0.5–1.5%). Direct antiglobulin test: negative with anti-IgA, G, M, D, E, positive with anti-complement antibodies. Identification of the antibodies: cold agglutinins and haemolysins of specificity anti-I.

The titre of the cold agglutinin was more than 1/500. The concentration of complement component C_3 was decreased. Immunoelectrophoretic pattern: IgA 100 IU/ml (n 40–225), IgM 150 IU/ml (n 45–335), IgG 60 IU/ml (n 65–200); IgM consisted of a linear band, as seen with paraprotein. Immunofluorescence pattern of the bone marrow: IgA 22%, IgM 24%, IgG 33%, kappa 70%, lambda 30% (normal). Reaction of Wassermann, VDRL and Paul Bunnell were negative. Erythrocyte enzyme patterns normal. Vitamin B_{12} concentration 315 pg/ml (normal).

On the basis of these findings the diagnosis of heterozygous β-thalassaemia combined with cold agglutinin disease was established. We slowly diminished and finally stopped prednisone without precipitating any acceleration of the anaemia. The patient returned to Suriname because he felt the cold Dutch weather was the cause of his troubles.

Comment

This patient is suffering from β-heterozygous thalassaemia and cold agglutinin disease. The two types of anaemia appear to be unrelated.

Received: September 20, 1977
Accepted: October 5, 1978

M. E. L. v. d. Burg,
Department of Internal Medicine,
Municipal Hospital, Bergweg,
Rotterdam (The Netherlands)

Table I. Coagulation studies

Test	Patient	Normal values
Bleeding time, min	8–15	4–7
Clotting time, min	7	5–8
Platelet count, mm³	30–40 × 10³	150–350 × 10³
PTT sec	49–55	30–45
PT sec	13	12–14
Fibrinogen, mg%	218	200–400
Factor VIII, %	90	50–150
Factor IX,	100	50–150
Factor XI	50	50–150

Table II. PTT of mixtures of patient's and normal plasma

Volumes of plasma		PTT sec	
normal	patient	immediate	after 1 h[1]
10	0	37	39
9	1	43	48
0	10	49	55
1	9	50	54

[1] After 1 h of incubation at 37 C.

tients. Factor XI was measured in 1 patient only and was normal [1]

These authors [1] suggested that the prolongation of PTT might be due to an interference with the action of a certain procoagulant, possibly a sphingolipid. As presently shown, the patient's plasma brought only a slight prolongation of the PTT of normal plasma. These results could suggest that our patient's plasma contains indeed a factor(s) responsible for the prolongation of PTT of normal plasma.

The biochemical disorder in Gaucher's disease results in excessive accumulation of sphingolipids in the reticuloendothelial system and according to *Dawson and Oh* [2] sometimes also in the plasma. It has already been shown that sphingolipids, particularly cerebrosides, inactivate or interfere with the action of procoagulants [3] The mechanism of this anticoagulant activity of sphingolipids is yet unclear according to *Hecht et al* [3] the inhibition of platelet factor 3 as well as other coagulation factors, may be responsible for this phenomenon Coagulation abnormalities have been found in sea blue histocytosis, a disorder in which phospholipids, particularly sphingolipids, accumulate in the reticuloendothelial system [1]

The question whether sphingolipids act as anticoagulants and interfere with the procoagulant activity and the prolongation of PTT warrants further investigations.

References

1 Bolkan, B. F and Sawitsky A. Factor IX deficiency in Gaucher disease. A *in vitro* phenomenon. Archs intern. Med. *136*, 489–492 (1976).
2 Dawson, G and Oh, J Y. Blood glucosylceramide levels in Gaucher's disease and its distribution amongst lipoprotein fractions. Clinica chim. Acta *75* 149–153 (1977).
3 Hecht, E Landburu, R. H and Seegers, W H. Sphingosine as an inhibitor of prothrombin activation with thromboplastin or with threone. Am. J Physiol. *189* 203–208 (1957).
4 Proctor R. R. and Rapaport, S. L. The partial thromboplastin time with kaolin, a simple screening test for first stage plasma clotting factor deficiencies. Am. J clin. Path. *36*. 212–219 (1961).
5 Rapaport, S. I Schiffman, S., and Patch, M. L. A simple specific one stage assay for plasma thromboplastin antecedent activity J Lab. clin. Med. *57* 771–776 (1976).

Received June 27 1978
Accepted. October 8, 1978

Prof. J Pinkhas, Department of Medicine 'D
Tel Aviv University Medical School,
Beilinson Medical Center Petah Tikva (Israel)

Original Papers

Acta haemat. 61 237–242 (1979)

Drug-Induced Kinetic Perturbations of the Marrow Blasts in Acute Leukemia. Effects of the Daunorubicin, Cytosine Arabinoside and 6-Thioguanine Combination

S. Amadori, M. C. Petti, C. Mastrovincenzo and M. G. Testa

Institute of Hematology, University of Rome, Rome

Key Words. Acute nonlymphocytic leukemia · Cell kinetics · Chemotherapy

Abstract. Cell kinetic changes induced in the marrow blasts by treatment with a triple cytotoxic regimen including daunorubicin, cytosine arabinoside and 6-thioguanine (DAT) were investigated in 6 previously untreated acute nonlymphocytic leukemia patients. A decrease in the labeling and mitotic indices was consistently observed 24 h after administration of daunorubicin, suggesting a G_2 block and a preferential lytic effect on the S-phase cells operated by the drug. Conversely, cytosine arabinoside and 6-thioguanine in combination induced a series of kinetic perturbations variable from case to case, however, three principal patterns of kinetic response were recognized and discussed in detail. Useful information for the planning of a more rational antileukemic therapy can be drawn from a systematic study of the kinetic effects induced by drug combinations.

Numerous experimental studies have clearly shown, in animal tumors, the possibility of significantly improving therapeutic response by selecting and scheduling the cytotoxic agents on the basis of cell kinetic considerations [4, 7, 8, 11]. Similar demonstration of superiority of the kinetically-based *antiblastic therapy* has not yet been given in man; consequently, antileukemic strategy continues in practice to be guided by empiric principles essentially derived from clinical observations.

We wish in this paper to report on the cytokinetic effects induced by the triple combination of daunorubicin, cytosine arabinoside and 6-thioguanine (DAT) on the marrow blasts of 6 patients affected by acute nonlymphocytic leukemia (ANLL).

Material and Methods

The study comprises 6 previously untreated ANLL patients, whose principal clinical and hematological characteristics at diagnosis are illustrated in table I. As induction treatment, all patients were given the DAT regimen consisting of the cyclic administration of daunorubicin (DNR), cytosine arabinoside (CA) and 6-thioguanine (TG) as set out in table II. Immediately before initiation of treatment and successively at fixed intervals, sam-

Varia

Arthur-Pappenheim-Preis 1978

Anlässlich der 24. Jahrestagung der Deutschen und Österreichischen Gesellschaft für Hämatologie und Onkologie wurde im Oktober 1978 in Göttingen der von den Nordmark Werken jährlich unterstützte Arthur Pappenheim-Preis verliehen.

Preisträger ist Herr Privatdozent Dr. med. *W. Tillmann*, Universitäts-Kinderklinik, Göttingen. Der Titel der prämierten Arbeit lautet: Beziehungen zwischen Stoffwechsel und Flexibilität von Erythrozytenmembranen.

Postgraduate Course in Genetic Red Blood Cell Defects
San Francisco, March 9–10, 1979

Under the direction of Dr. L. E. Lie-Injo and the University of California San Francisco School of Medicine Extended Program in Medical Education, a postgraduate course in Genetic Red Blood Cell Defects will be held.

Dr. T. B. Bradley will lecture on structure-function relationship of abnormal hemoglobins, Dr. Y. W. Kan on the molecular basis of the thalassaemia, Dr. B. H. Lubin on pathogenesis of sickle cell anemia, Dr. W. C. Valentine on genetic red blood cell enzyme deficiencies and hemolysis, Dr. S. B. Shohet on membrane abnormalities, Dr. Y. W. Kan on prenatal diagnosis, Dr. W. C. Mentzer on clinical aspects and management of sickle cell anemia and thalassaemia, Dr. F. B. Livingstone on dynamics and epidemiology of the genetic red blood cell defects, Dr. A. J. Ammann on genetic red blood cell defects and infections, Dr. L. E. Lie-Injo on genetic red blood cell defects and severe neonatal jaundice, Dr. A. W. Nienhuis on chelating agents and Dr. C. J. Epstein and Dr. R. Davis on genetic counseling.

For further particulars please contact Ms. Veronica Galusha, School of Medicine Extended Program in Medical Education, University of California, San Francisco, CA. 94143 (USA).

Announcement

The fourth annual 'Advanced Institute in Methods of Immunological Diagnosis' conducted under the auspices of the World Health Organization Collaborating Laboratory for the Serology of Autoimmune Diseases at Wayne State University School of Medicine, will be held June 17–23, 1979. The course is to be held on the wooded campus of Cranbrook Educational Community in Bloomfield Hills, Mich.

This year the course will emphasize selected, newer methods applicable to the diagnosis of human diseases.

Category 1 credit for postgraduate medical education has been approved. Deadline for receiving applications is April 15. Further information can be obtained by writing to: Noel R. Rose, MD, PhD, Department of Immunology and Microbiology, Wayne State University School of Medicine, 540 East Canfield, Detroit MI 48201 (USA).

Original Papers

Acta haemat. 61: 237–242 (1979)

Drug-Induced Kinetic Perturbations of the Marrow Blasts in Acute Leukemia. Effects of the Daunorubicin, Cytosine Arabinoside and 6-Thioguanine Combination

S. Amadori, M. C. Petti, C. Mastrovincenzo and M. G. Testa
Institute of Hematology, University of Rome, Rome

Key Words. Acute nonlymphocytic leukemia · Cell kinetics · Chemotherapy

Abstract. Cell kinetic changes induced in the marrow blasts by treatment with a triple cytotoxic regimen including daunorubicin, cytosine arabinoside and 6-thioguanine (DAT) were investigated in 6 previously untreated acute nonlymphocytic leukemia patients. A decrease in the labeling and mitotic indices was consistently observed 24 h after administration of daunorubicin, suggesting a G_2 block and a preferential lytic effect on the S-phase cells operated by the drug. Conversely, cytosine arabinoside and 6-thioguanine in combination induced a series of kinetic perturbations variable from case to case; however, three principal patterns of kinetic response were recognized and discussed in detail. Useful information for the planning of a more rational antileukemic therapy can be drawn from a systematic study of the kinetic effects induced by drug combinations.

Numerous experimental studies have clearly shown, in animal tumors, the possibility of significantly improving therapeutic response by selecting and scheduling the cytotoxic agents on the basis of cell kinetic considerations [4, 7, 8, 11]. Similar demonstration of superiority of the kinetically based antiblastic therapy has not yet been given in man; consequently, antileukemic strategy continues in practice to be guided by empiric principles essentially derived from clinical observations.

We wish in this paper to report on the cytokinetic effects induced by the triple combination of daunorubicin, cytosine arabinoside and 6-thioguanine (DAT) on the marrow blasts of 6 patients affected by acute nonlymphocytic leukemia (ANLL).

Material and Methods

The study comprises 6 previously untreated ANLL patients, whose principal clinical and hematological characteristics at diagnosis are illustrated in table I. As induction treatment, all patients were given the DAT regimen consisting of the cyclic administration of daunorubicin (DNR), cytosine arabinoside (CA) and 6-thioguanine (TG) as set out in table II. Immediately before initiation of treatment and successively at fixed intervals, sam-

Table I. Main clinical and laboratory findings of patients

Patient	Age years	Sex	Type of leukemia	Blasts in blood n/mm³	Blasts in marrow %
Z.R.	33	M	AML	122,120	90
P A	18	M	AMoL	34,800	90
S.R.	19	M	AMML	15,386	90
L.T	35	F	AMML	7,560	71
F F	30	M	AMML	55 440	80
O E.	52	M	AMML	19,656	80

AML = Acute myelocytic leukemia AMoL = acute monocytic leukemia AMML = acute myelomonocytic leukemia.

Table II. Outline of the DAT regimen

Drug	Dose	Schedule[1]
DNR	2 mg/kg, i.v	day 1
CA	1.5 mg/kg, i.v., q 12 h	days 2–6
TG	1.5 mg/kg, p.o., q 12 h	days 2–6

[1] Course repeated after 10 days of rest for 3 cycles.

ples of marrow blood (2 ml) were aspirated into a heparinized syringe to determine the mitotic index and the *in vitro* ³H-thymidine labeling index of the leukemic cells. The mitotic index (MI) was evaluated by directly enumerating mitotic figures in at least 3,000 leukemic blasts after May Grünwald-Giemsa staining; results are expressed as per thousand. The labeling index (LI) was evaluated autoradiographically by incubating a portion of the aspirate for 1 h at 37°C with ³H-thymidine (³H TdR, specific activity 2 Ci/mmol, 4–5 μCi/ml of marrow). After fixation in absolute methyl alcohol for 15 min, the smears were dipped in Kodak NTB 2 emulsion, exposed at 4°C for 8–10 days, developed in Kodak D19 and stained with Giemsa. The percentage of labeled cells (5 or more grains) was determined by counting 1,000–3,000 leukemic blasts.

Results

The effects of treatment with the DAT regimen on the MI and LI of leukemic marrow blasts are reported in tables III and IV In all cases a decrease in the MI 24 h after DNR was observed. The fall in the MI was accompanied by a parallel decline in the LI in all the patients with the exception of patient F F in whom, on the contrary a significant increase at the 24th hour was recorded. During treatment with the combination CA+TG the dynamic perturbations of the two kinetic parameters appeared rather variable in the patients on study It was possible, however to recognize 3 different modalities of response (1) Patients P A. and O E. showed a progressive increase in the LI not accompanied by appreciable variations in the MI which remained around zero value for the entire treatment. Between 48 and 72 h after the end of chemotherapy the LI began to slow down in both cases, being accompanied in patient P A by a significant return of the mitotic activity (fig. 1) (2) In patients L. T and Z. R., on the other hand, the rise in the LI occurred in a more irregular manner with a clearly oscillating evolution accompanied by semi-synchronous variations in the MI (fig. 2) In these 2 cases too it was possible to observe after therapy a return of the kinetic parameters towards their baseline values with, however a more irregular evolution particularly evident in patient L. T., in whom 2 consecutive waves of proliferation were observed at the 168th and 240th hour (3) The kinetic response of patient S. R. appeared completely different (fig. 3) For the entire duration of treatment MI and LI remained highly depressed without appreciable variations. However 24 h after cessation of ther

Table III. Changes in the labeling index (LI %) of marrow blasts induced by DAT

Patient	Hours from initiation of DAT										
	0	24	48	72	96	120	144	168	192	240	384
Z.R.	3	2.2	4.2	0	3.3	0.6	–	–	–	–	–
P.A.	0.3	0.03	0.02	0	2.4	5 4	–	–	7	4.7	3.7
S.R.	1.9	0 6	0.16	0.3	0.1	0.5	0.16	5 7	2.1	0.05	0.55
L.T	6.5	0.1	4.3	5.4	0 03	10.2	2.2	4.2	1.2	9.8	4 1
F.F	9 4	18.2	3.9	1 1		–	–	–	–	–	
O.E.	15	4 4	8.5	11.4	17.8	14.7	–	17.3	9.5		

Table IV Changes in the mitotic index (MI %o) of marrow blasts induced by DAT

Patient	Hours from initiation of DAT										
	0	24	48	72	96	120	144	168	192	40	384
Z.R.	3.3	0.3	0	0	6	4	4	0.5	2	1	–
P.A.	6	0	0	0	0	0	–	–	0	2	0
S.R.	2.6	0	0	0	0	0	0	1	1.5	0	0
L.T	10.1	0	0	4	0	2	2	5	0	4	–
F.F	2	0.3	0.3	0.3			–			–	–
O.E.	3	2	0	0		0	–	0	0		–

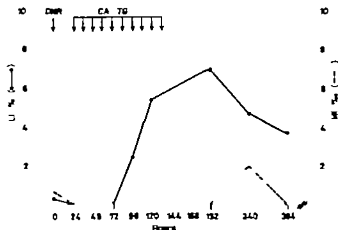


Fig. 1. Patient P A. Variations in labeling and mitotic indices of marrow blasts during treatment with DAT

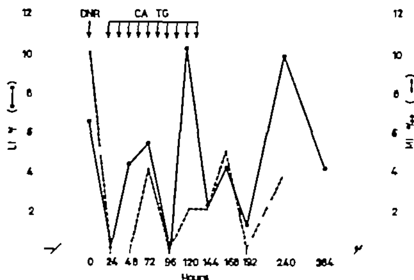


Fig. 2. Patient L.T. Variations in labeling and mitotic indices of marrow blasts during treatment with DAT

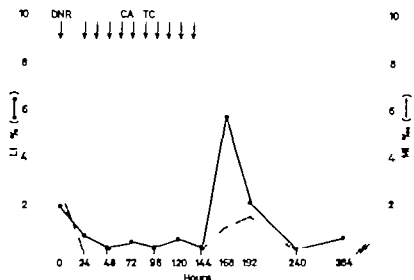


Fig. 3. Patient S.R. Variations in labeling and mitotic indices of marrow blasts during treatment with DAT

apy a rapid and significant increase in the LI took place accompanied by a semisynchronous inflection in the MI. In this case also the wave of proliferation was followed by a gradual return towards pretreatment levels. Analysis of the drug-induced kinetic changes was difficult in patient F.F. because of the brevity of the period of study. After the initial increase at the 24th hour we observed, with the starting of the CA+ TG combination, a profound decline in the LI while, after its initial fall, the MI remained at near zero values throughout the study period. Although no attempt has been made to quantify the changes in marrow cellularity during DAT treatment, we were able, however to record in all cases a gradual reduction of the leukemic infiltration as well as of the absolute number of peripheral blasts. As for the prognostic value of the DAT-induced kinetic changes, no relationship between cytokinetic effects and thera-

peutic response was apparent in this small series of ANLL patients. Excluding patient F F who died early during induction from sepsis, complete remission was in fact achieved in all the cases except P A.

Discussion

Detailed studies of the cytokinetic effects induced by drug combinations on leukemic cells in man are rather scanty in literature in particular in the case of the DAT regimen they are completely lacking. The results we obtained in 6 patients affected by ANLL confirm, first of all, the well-known cytokinetic effects of DNR [1 9 10] Trapping of proliferating cells in G_2 is, in fact, suggested by the rapid decline in the MI observable in all the patients. Similarly the fall in the LI evident in 5 cases 24 h after DNR seems to be in relation to the G -S block induced by the drug as well as to its cytocidal action, particularly active on the S-phase cells. A significant increase in the LI has been reported by *Stryckmans et al.* [10] in a patient affected by ANLL, beginning 48 h after administration of DNR. This phenomenon was interpreted by the authors as evidence of a recruitment in cycle of resting cells secondary to the drastic reduction of the leukemic mass operated by the drug. A similar mechanism could explain the sharp increase in the LI observed in patient F F 24 h after DNR. The precocity of the phenomenon, however seems to favor the recruitment in cycle of resting cells stationating near the G -S boundary rather than in G [2] The CA+ TG combination determined, in the 5 patients adequately studied, a series of cytokinetic perturbations variable from case to case, but which can be classified into three principal types (1) A significant and progressive rise in the LI not accompanied by variations in the MI, which remained at zero values for the entire duration of therapy Such dynamic behavior of the kinetic parameters appears related to a progressive accumulation of cycling cells in S, probably induced by CA [3] The cells, however because of the combined cytotoxic effect of CA and TG became unable to complete DNA synthesis and proceed therefore towards mitosis. The decline in the LI starting 48 h after the end of chemotherapy associated with the reappearance of mitotic figures, signaled the renewal of the proliferative activity in a semisynchronous fashion by a cohort of cells escaping the cytotoxic action of the CA+ TG combination and, therefore, probably resting in a quiescent state during therapy (2) Wide oscillations in the LI, accompanied by almost synchronous modifications of the MI In this case, the dynamic evolution of the kinetic parameters could be explained by successive accumulations of cells in S phase probably induced by the intermittent administration of CA. However the cells appeared capable to complete DNA synthesis and, therefore, proceed in a semisynchronous manner towards mitosis thus escaping, at least in part, the cytotoxic effect of TG (3) Almost complete suppression during treatment of any proliferative activity with rapid reappearance of a semisynchronous wave of proliferation immediately after the end of therapy A double mechanism seems to be operative firstly a near complete killing of all cells in S brought about by the combined cytotoxic effect of CA and TG secondly a reduction of the G_1 S cellular flow secondary to the cycle-specific action of TG [5 6] In conclusion, the evident individual variability of

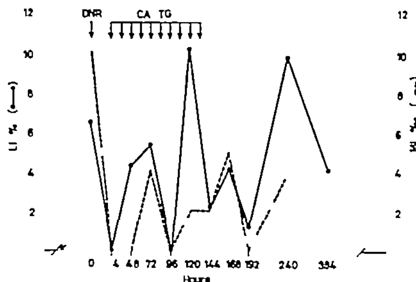


Fig. 2. Patient L.T Variations in labeling and mitotic indices of marrow blasts during treatment with DAT

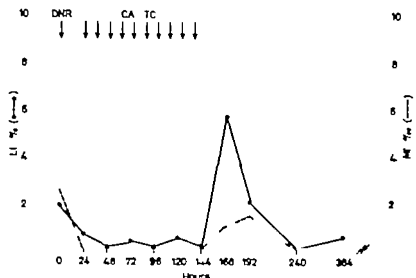


Fig. 3. Patient S.R. Variations in labeling and mitotic indices of marrow blasts during treatment with DAT

apy a rapid and significant increase in the LI took place accompanied by a semisynchronous inflection in the MI In this case also, the wave of proliferation was followed by a gradual return towards pretreatment levels. Analysis of the drug-induced kinetic changes was difficult in patient F F because of the brevity of the period of study After the initial increase at the 24th hour we observed, with the starting of the CA+ TG combination a profound decline in the LI while, after its initial fall, the MI remained at near zero values throughout the study period. Although no attempt has been made to quantify the changes in marrow cellularity during DAT treatment, we were able, however to record in all cases a gradual reduction of the leukemic infiltration as well as of the absolute number of peripheral blasts. As for the prognostic value of the DAT induced kinetic changes, no relationship between cytokinetic effects and thera-

Acta haemat. 61: 243–250 (1979)

A Modified Assay for Studies of Cultured Granulocyte Precursors Cryopreservation of Stimulating Mononuclear Cells

Ranko Odavic, Johanna Blom and Eugene A. Beck

Central Hematology Laboratory, Inselspital, Berne

Key Words. Human peripheral blood · Mononuclear cells · Cryopreservation · Bone marrow culture · Colony-stimulating factor

Abstract. Mononuclear cells (MNC) isolated from peripheral blood of healthy donors, were cryoprotected by dimethyl sulfoxide and stored in liquid nitrogen. Colony-stimulating factor (CSF), produced by cryopreserved MNC, was compared with that of nonfrozen controls in a double-layer agar system with human bone marrow as target cells. Our results indicate that cryopreserved MNC retain their ability to stimulate myelopoiesis-committed stem cells after freezing. In addition, evidence was obtained that CSF of feeder layers changes, depending on the duration of preincubation and cell concentration. In a system where either stimulating or target cells are cryopreserved the dynamics of interactions between normal or abnormal cell lines can thus be studied.

Introduction

The proliferation and differentiation of myelopoiesis-committed progenitor cells (colony-forming units in culture, CFU-c), immobilized in agar or methyl cellulose, results in colonies which can be enumerated and studied morphologically. The number and size of the formed colonies depends critically on the presence of a class of stimulators, tentatively designed as colony-stimulating factor(s) or activity (CSF or CSA). Different sources of CSF have been described which stimulate proliferation of human CFU-c *in vitro*, e.g. human embryo kidney cells [4], embryo fibroblasts [10], human spleen cells [30], pulmonary macrophages [13], monkey lung cells [34], vascular endothelium cells [18], adipose tissue, skeletal muscle and peritoneum [19] and placenta [5]. The most commonly used stimulators are, however, human peripheral blood leukocytes. These cells are used either for preparation of feeder layers [32] or of conditioned media [17]. On average, feeder layers provide a higher stimulation than conditioned medium [35, 39].

The classical double-layer technique for cloning of human CFU-c [32] has found wide clinical application [15, 29, 37]. We

the cytokinetic response of the leukemic blasts after treatment with the DAT regimen, clearly underlines the difficulty of extrapolating directly from this type of study useful information for the elaboration of more effective combination chemotherapy. Therapeutic response depends, apart from cell kinetics, on many other biological factors (biochemical sensitivity of leukemic cells, dose, transport, metabolism of the drugs used, etc.) a knowledge of which seems of fundamental importance for a better design of the therapeutic strategy. There is no doubt, however, that analysis of the kinetic changes induced in the leukemic clone by drug combinations will, in union with appropriate information on the perturbed behavior of the normal cell compartments, contribute to providing the basis for a more rational approach to leukemia therapy.

References

1 Ernst, P.: Perturbation of generation cycle of human leukemic blast cells *in vivo* by daunomycin. Scand. J. Haematol. *11*: 13–22 (1973).
2 Gelfant, S.: A new concept of tissue and tumor cell proliferation. Cancer Res. *37*: 3845–3862 (1977).
3 Killmann, S. A.: Effect of cytostatic drugs on the kinetics of leukemic blast cells in man. Schweiz. med. Wschr. *104*: 278–284 (1974).
4 Klein, H. O. and Lennartz, K. J.: Chemotherapy after synchronization of tumor cells. Semin. Hemat. *11*: 203–227 (1974).
5 Le Page, G. A. and White, S. C.: Scheduling of arabinosylcytosine and 6-thioguanine therapy. Cancer Res. *33*: 946–951 (1973).
6 Nelson, J. A., Carpenter, J. W., Rose, L. M., and Adamson, D. J.: Mechanisms of action of 6-thioguanine, 6-mercaptopurine and 8-azaguanine. Cancer Res. *35*: 2872–2878 (1975).
7 Silvestrini, R., Di Marco, A., Di Marco, S., and Dasdia, T.: The action of daunomycin on the metabolism of nucleic acids of normal and neoplastic cells growing *in vitro*. Tumori *49*: 399–412 (1963).
8 Skipper, H. E., Schabel, F. M., Mellett, L. B., Montgomery, J. A., Wilkoff, L. J., Lloyd, H. H., and Brockman, R. W.: Implications of biochemical, cytokinetics, pharmacologic and toxicologic relationship in the design of optimal therapeutic schedules. Cancer Chemother. Rep. *54*: 431–450 (1970).
9 Stryckmans, P. A. and Manaster, J.: Kinetic aspects of leukaemia therapy; in Staquet, The design of clinical trials in cancer therapy, p. 152 (Editions Scientifiques Européennes, Brussels 1972).
10 Stryckmans, P. A., Manaster, J., Lachapelle, F., and Socquet, M.: Mode of action of chemotherapy *in vivo* on human acute leukemia. I. Daunomycin. J. clin. Invest. *52*: 126–133 (1973).
11 Wiernik, P. H., Schimpff, S. C., Schiffer, C. A., Lichtenfeld, J. L., Aisner, J., O'Connell, M. J., and Fortner, C.: Randomized clinical comparison of daunorubicin (NSC-82151) alone with a combination of daunorubicin, cytosine arabinoside (NSC-63878), 6-thioguanine (NSC 752), and pyrimethamine (NSC 3061) for the treatment of acute nonlymphocytic leukemia. Cancer Treat. Rep. *60*: 41–53 (1976).

Received: July 18, 1978
Accepted: October 5, 1978

S. Amadori, MD, Institute of Hematology, University of Rome, Via Lancisi 3, I-00161 Rome (Italy)

tion of hemolysate enhances the activity of low level CSF [3, 12, 16] we prepared feeder layers containing fresh hemolysate. This addition did not influence the CSF of fresh feeder layers (76.7 colonies without, and 78.6 colonies with hemolysate, n = 11). In the presence of hemolysate, the stimulation of frozen target cells by cryopreserved MNC was enhanced in some experiments, especially in cultures with low colony counts, but unchanged in others. Therefore, hemolysate was omitted in subsequent experiments.

Dicke et al. [11] observed recently that the CSF of feeder layers containing fresh leukocytes varies depending on the duration of preincubation before the actual test. Considering the importance of this finding for clinical application of the double-layer technique we decided to study this variable in more detail. Peripheral MNC from healthy donors were divided into two parts. The first part was separated into aliquots and frozen. After 2 h storage in liquid nitrogen the cells were thawed and washed free of cryoprotector. Feeder layers, containing either fresh (nonfrozen) or frozen MNC, could thus be prepared on the same day (day 0). On several subsequent days, frozen bone marrow cells were thawed and plated in triplicate on preincubated feeder layers. Colonies were counted 12 days after plating. A considerable change of CSF production was indeed observed with a maximum around day 5 of feeder layer preincubation (fig. 1).

In a further experiment, varying amounts of fresh bone marrow cells (0.5–3.0 × 10⁴ cells/ml) were plated on feeder layers made from fresh or frozen peripheral MNC as previously described. Bone marrow cells, as well as MNC, were obtained from

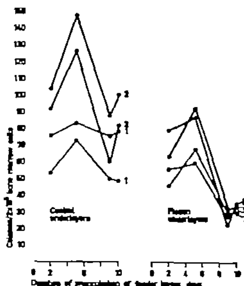


Fig. 1. Change of CSF expressed as number of granulocyte colonies and depending on preincubation of feeder layers. The curves connecting open or closed circles correspond to two different preparations of MNC. The numbers 1 and 2 correspond to cryopreserved bone marrow (target cells) from two different donors. Control under layers consisted of fresh, i.e. nonfrozen MNC. Frozen MNC were thawed and feeder layers preincubated for varying periods of time (see text).

single donors. In this particular experiment, feeder layers had been incubated for 4 days prior to plating. Colonies were again counted after 12 days. The results shown in figure 2 suggest that, at least in the tested range, a direct linear correlation exists between the number of target cells and colony counts.

Most authors using the double-layer technique operate with feeder layers containing a standard amount of stimulating cells, i.e. 1 × 10⁶ cells/ml [32]. Recently this amount of *fresh* leukocytes in feeder layers has been confirmed as being optimal

tested the possibility of using cryopreserved peripheral mononuclear cells (MNC) for the preparation of feeder layers. A constant supply of frozen stimulating cells could reduce the variability of CSA in the assay. Our results indicate that such an approach is indeed possible. Additional evidence was obtained that CSA of feeder layers changes as a function of preincubation time and cell concentration.

Materials and Methods

Isolation of MNC from Peripheral Blood

Red blood cells and granulocytes were separated from MNC by the method of *Bøyum* [2]. Concentrated MNC were washed once with Hank's balanced salt solution containing 10% fetal calf serum (FCS), but no calcium or magnesium ions. Washed cells were centrifuged at 750 *g* for 10 min and resuspended in McCoy 5A modified medium containing 20% FCS.

Preparation of Target Cell Suspensions

Human bone marrow was aspirated during general anesthesia from hematologically normal patients undergoing surgery for sciatic nerve compression. Informed consent was obtained from each patient. Bone marrow aspirate was anticoagulated with preservative-free heparin (10 U/ml) and passed through a 20 gauge needle. The cell suspension was centrifuged at 750 *g* for 10 min. MNC were separated from the resulting buffy coat by density centrifugation [2] and after washing resuspended in medium containing 20% FCS.

Cryopreservation

To the cell suspensions, containing 10–20 × 10⁶ cells/ml and cooled in an ice bath, dimethyl sulfoxide was added slowly up to 10% v/v final concentration. Sealed ampoules were frozen at a controlled rate of 1–2 °C/min using a programmed freezing machine (Planer, Sunbury, England). After storage in liquid nitrogen for various periods of time the cells were quickly thawed at 37 °C, diluted 10-fold during 30 min with medium containing 20% FCS, centrifuged at 750 *g* for 5 min, and resuspended in the original volume of medium and FCS. Cell clumps were dispersed with DNA-ase (Serva, Heidelberg, FRG).

Preparation of Hemolysate

In some experiments hemolysate was added to the feeder layers containing cryopreserved MNC. Human erythrocytes were washed five times with saline and lysed by the addition of an equal volume of distilled water. After standing at 4 °C for 1 h and 15 min centrifugation at 1,000 *g*, 0.05 ml of the supernatant was added to 1 ml of cell suspension used for preparation of feeder layers. Final hemoglobin concentration was calculated to be 5 mg/ml.

Bone Marrow Culture in vitro

The level of CSF produced by nonfrozen or cryopreserved MNC was measured by the double-layer agar technique described by *Pike and Robinson* [32]. Feeder layers were prepared by immobilizing 1 × 10⁶ MNC in 1 ml of 0.5% agar unless stated otherwise. Upper layers consisted of 1 or 2 × 10⁵ bone marrow cells, suspended in 1 ml of McCoy 5A modified medium containing 20% FCS and 0.25% agar. Incubation lasted for 10–12 days at 37 °C in a fully humidified atmosphere with 5% CO_2. Colonies containing more than 50 cells were counted using an inverted microscope.

Results

MNC, isolated from human peripheral blood were used for preparation of feeder layers, either fresh (nonfrozen) or after storage in liquid nitrogen for at least 2 h. No consistent difference in colony growth was found when pairs of nonfrozen and frozen feeder layers were tested with *fresh* bone marrow target cells. When, however, 41 samples from *cryopreserved* bone marrow were used to test the CSF of feeder layers much lower colony counts were obtained with frozen MNC (mean, 47.8 colonies) than with fresh MNC (mean 81.6 colonies). Based on published evidence that the addi

ties characteristic for biological techniques. The results are influenced by such factors as the quality of the serum, conditions of incubation, composition of the cell population plated and, last but not least, the amount and quality of stimulation.

According to the published evidence [35 39] feeder layers provide a much higher stimulation than conditioned media. This difference can be attributed, in part, to a lower concentration of CSF in conditioned medium. On the other hand, numerous variables are influencing the activity of feeder layers, e.g. as yet poorly defined interindividual variability cyclic changes and effects of diseases, such as viral infections [24] With the intention of reducing the variability of feeder layers and providing a potent, reproducible stimulation we studied the CSF production by MNC after cryopreservation.

Ragab et al. [33] reported recently on CSF production by peripheral blood leukocytes from one normal donor after cryopreservation. These authors obtained less stimulation with cryopreserved cells than with fresh (nonfrozen) leukocytes, using bone marrow from 16 children with ALL in remission as target cells. Nonseparated leukocytes used in their study are, however more susceptible to freezing injury than concentrated MNC [9]

CSF production of frozen or nonfrozen MNC in feeder layers, tested with fresh target cells, was comparable. However when frozen target cells were used we found much lower stimulation by cryopreserved MNC. Feeder layers, used in our experiments, contained primarily lymphocytes and monocytes. Both cell types produce CSF according to numerous publications [6, 8 14 27 31 36] but the main function of regulating myelopoiesis seems to reside with monocytes [21] Since monocytes are quite susceptible to freezing injury [9] it is possible that feeder layers prepared from frozen cells contained less functionally intact monocytes than nonfrozen cell suspensions. Results of several authors suggest that the low CSF or 'freezing injury' could be corrected by adding hemolysate [3 12, 16] or fresh plasma, respectively serum [7 23] to cell cultures. Our own results on effects of hemolysate were inconclusive. Thus, we decided against introduction of variables, in hemolysate or plasma, which were not essential for cell growth.

The production of CSF is an active metabolic process requiring viable cells and protein synthesis [22] CFU-c are, on the other hand, dependent on the presence of CSF during the whole period of proliferation [25 26] This can explain the observed dynamics of CSF production as a function of the preincubation period. Several days may be necessary to activate the monocyte macrophage system, and for these cells to produce CSF CFU-c also have to pass through a quiescent period before they 'burst' into proliferation around day 5 as shown in kinetic studies using ^{3}H-thymidine [1 28, 38]. A similar pattern of CSF production by nonfrozen leukocytes [11] and during preparation of conditioned media [e.g. 8 36] has been previously described. Cryopreserved MNC show the same fluctuation of CSF production. We conclude, therefore, that cryopreserved MNC produce CSF by the same active process as nonfrozen cells. Therefore, it is essential to standardize the time of preincubating feeder layers. A peak of stimulating activity may be expected between days 4 and 6 of incubation from fresh or cryopreserved MNC. The

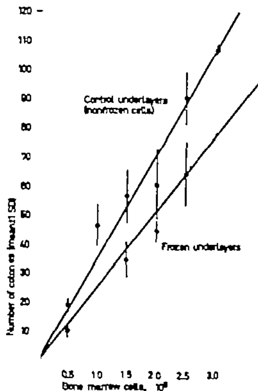


Fig. 2. Effects of nonfrozen or cryopreserved MNC in feeder layers on formation of granulocyte colonies. For this experiment varying numbers of nucleated bone marrow cells, obtained from a single, nonfrozen sample, were plated as indicated.

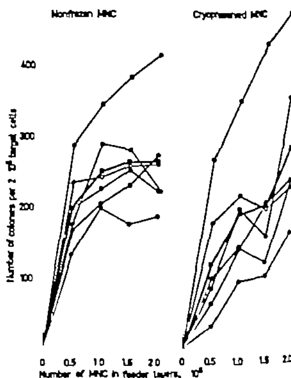


Fig. 3. Dependence of CSF on the number of *stimulating MNC. 0.5, 1.0, 1.5* and 2.0×10^6 fresh or cryopreserved MNC were used for preparation of feeder layers. After 4 days of preincubation their CSF production was tested with 2×10^5 of frozen human bone marrow target cells. Mean values of six experiments are represented by squares.

[19] We wanted to know whether similar results were obtained with *frozen* MNC and studied, therefore, a series of feeder layers containing variable amounts of either fresh or cryopreserved MNC. These feeder layers were tested with a constant number of bone marrow target cells. Figure 3 shows a few representative response curves which are nonlinear. At the highest concentration of MNC (2×10^6 cells/ml) an inhibiting effect, sometimes present with fresh stimulating cells, seems to be abolished by the process of freezing.

Finally a prerequisite for the use of cryopreserved MNC in feeder layers would be a reasonable reproducibility of CSF production by the same cells stored in different ampoules. An acceptable day-to-day reproducibility was obtained especially with high colony counts (relative standard deviation ~10%)

Discussion

The technique of cloning CFU-c in semisolid media enables the monitoring of the *quantity and proliferative activity of these* progenitor cells. The method is, however afflicted by all the limitations and difficul-

ties characteristic for biological techniques. The results are influenced by such factors as the quality of the serum, conditions of incubation, composition of the cell population plated and, last but not least, the amount and quality of stimulation.

According to the published evidence [35 39] feeder layers provide a much higher stimulation than conditioned media. This difference can be attributed, in part, to a lower concentration of CSF in conditioned medium. On the other hand, numerous variables are influencing the activity of feeder layers, e.g. as yet poorly defined interindividual variability cyclic changes and effects of diseases, such as viral infections [24]. With the intention of reducing the variability of feeder layers and providing a potent, reproducible stimulation we studied the CSF production by MNC after cryopreservation.

Ragab et al. [33] reported recently on CSF production by peripheral blood leukocytes from one normal donor after cryopreservation. These authors obtained less stimulation with cryopreserved cells than with fresh (nonfrozen) leukocytes, using bone marrow from 16 children with ALL in remission as target cells. Nonseparated leukocytes used in their study are, however more susceptible to freezing injury than concentrated MNC [9]

CSF production of frozen or nonfrozen MNC in feeder layers, tested with fresh target cells, was comparable. However when frozen target cells were used we found much lower stimulation by cryopreserved MNC. Feeder layers, used in our experiments, contained primarily lymphocytes and monocytes. Both cell types produce CSF according to numerous publications [6, 8, 14 27 31 36] but the main function of regulating myelopoiesis seems to reside with monocytes [21] Since monocytes are quite susceptible to freezing injury [9] it is possible that feeder layers prepared from frozen cells contained less functionally intact monocytes than nonfrozen cell suspensions. Results of several authors suggest that the low CSF or 'freezing injury' could be corrected by adding hemolysate [3 12, 16] or fresh plasma, respectively serum [7 23] to cell cultures. Our own results on effects of hemolysate were inconclusive. Thus, we decided against introduction of variables, in hemolysate or plasma, which were not essential for cell growth.

The production of CSF is an active metabolic process requiring viable cells and protein synthesis [22] CFU-c are, on the other hand, dependent on the presence of CSF during the whole period of proliferation [25 26] This can explain the observed dynamics of CSF production as a function of the preincubation period. Several days may be necessary to activate the monocyte-macrophage system, and for these cells to produce CSF CFU-c also have to pass through a quiescent period before they 'burst' into proliferation around day 5 as shown in kinetic studies using ^{3}H-thymidine [1 28, 38] A similar pattern of CSF production by nonfrozen leukocytes [11] and during preparation of conditioned media [e.g. 8, 36] has been previously described. Cryopreserved MNC show the same fluctuation of CSF production. We conclude, therefore, that cryopreserved MNC produce CSF by the same active process as nonfrozen cells. Therefore it is essential to standardize the time of preincubating feeder layers. A peak of stimulating activity may be expected between days 4 and 6 of incubation from fresh or cryopreserved MNC. The

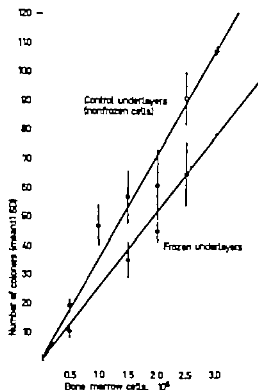


Fig. 2. Effects of nonfrozen or cryopreserved MNC in feeder layers on formation of granulocyte colonies. For this experiment varying numbers of nucleated bone marrow cells, obtained from a single, nonfrozen sample, were plated as indicated.

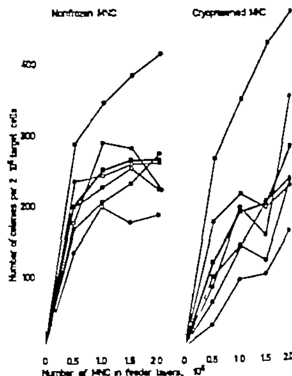


Fig. 3. Dependence of CSF on the number of stimulating MNC. 0.5 1.0, 1.5 and 2.0×10^6 fresh or cryopreserved MNC were used for preparation of feeder layers. After 4 days of preincubation their CSF production was tested with 2×10^5 of frozen human bone marrow target cells. Mean values of six experiments are represented by squares.

[19] We wanted to know whether similar results were obtained with *frozen* MNC and studied, therefore, a series of feeder layers containing variable amounts of either fresh or cryopreserved MNC. These feeder layers were tested with a constant number of bone marrow target cells. Figure 3 shows a few representative response curves which are nonlinear At the highest concentration of MNC (2×10^6 cells/ml) an inhibiting effect, sometimes present with fresh stimulating cells, seems to be abolished by the process of freezing.

Finally a prerequisite for the use of cryopreserved MNC in feeder layers would be a reasonable reproducibility of CSF production by the same cells stored in different ampoules. An acceptable day-to-day reproducibility was obtained especially with high colony counts (relative standard deviation ~10%)

Discussion

The technique of cloning CFU-c in semisolid media enables the monitoring of the quantity and proliferative activity of these progenitor cells. The method is, however afflicted by all the limitations and difficul-

pheral blood. J clin. Invest. *51* 2981 2983 (1972).

15 Goldman, J M. and Sultan, C.. Clinical applications of bone marrow culture. Lancet *ii*. 696–698 (1975).

16 Holt, W Kern, P Kubanek, B., and Heimpel, H.. Some factors influencing granulocytic colony formation *in vitro* by human white blood cells. Blood *44* 511 515 (1974).

17 Iscove, N N., Senn, J S Till, J E., and McCulloch, E. A.. Colony formation by normal and leukemic human marrow cells in culture: effect of conditioned medium from human leukocytes. Blood *37* 1–5 (1971).

18 Knudtzon, S. and Mortensen, B. T Growth stimulation of human bone marrow cells in agar culture by vascular cells. Blood *46*. 937–943 (1975).

19 Knudtzon, S. and Hertz, W Human tissues as source of colony-stimulating factor in human bone marrow cultures. Scand. J Haematol. *19* 482–486 (1977).

20 Kurland, J and Moore, M. A. S.. Modulation of hemopoiesis by prostaglandins. Expl. Hematol., Copenh. *5* 357–373 (1977).

21 Kurland, J and Moore, M. A. S.. The regulatory role of the macrophage in normal and neoplastic hemopoiesis; in Baum and Ledney Experimental hematology today pp. 51–62 (Springer, New York 1977)

22 Lan, L. McCulloch, E. A Till, J E., and Price, G B.. The production of hemopoietic growth factors by PHA-stimulated leukocytes. Expl. Hematol., Copenh. 6. 114–121 (1978).

23 Mangi, R. J and Mardiney M. R.. The *in vitro* transformation of frozen-stored lymphocytes in the mixed lymphocyte reaction and in culture with phytohemagglutinin and specific antigens. J exp. Med. *132*. 401–416 (1970).

24 Metcalf, D. and Wahren, B.. Bone marrow colony-stimulating activity of sera in infectious mononucleosis. Br med. J *iii* 99–101 (1968).

25 Metcalf, D. and Moore, M. A. S Haemopoietic cells, p. 391 (North-Holland, Amsterdam 1971).

26 Metcalf, D.. Hemopoietic colonies, p. 18 (Springer Berlin 1977).

27 Moore, M. A. S., Williams, N., and Metcalf, D.: *In vitro* colony formation by normal and leukemic human hematopoietic cells: interaction between colony-forming and colony-stimulating cells. J natn. Cancer Inst. *50*· 591–602 (1973).

28 Morgan, D. A., McCredie, K. B and Durett, A. G.: Liquid culture assays of mouse bone marrow granulopoiesis. Expl. Hematol., Copenh. 2. 174–181 (1974).

29 Odavic, R.; Beck, E. A. and Bucher, U Zur klinischen Bedeutung des Nachweises von Granulozytenkolonien in Knochenmarkkultur Schweiz. med. Wschr *106*. 743–745 (1976).

30 Paran, M., Sachs, L., Barak, Y and Resnitzky P.: *In vitro* induction of granulocyte differentiation in hematopoietic cells from leukemic and non-leukemic patients. Proc. natn. Acad. Sci. USA *67* 1542 1549 (1970).

31 Parker J W and Metcalf, D.. Production of colony-stimulating factor in mixed leucocyte cultures. Immunology 26. 1039–1049 (1974).

32 Pike, B. L. and Robinson, W A.. Human bone marrow colony growth in agar-gel. J cell. Physiol. 76. 77–84 (1970).

33 Ragab, A. H., Gilkerson, E., and Myers, M.: Factors in the cryopreservation of bone marrow cells from children with acute lymphocytic leukemia Cryobiology *14* 125–134 (1977).

34 Ratzan, J Moore, M. A. S and Yunis, A. A.. Effect of chloramphenicol-thiamphenicol on the *in vitro* colony-forming cells. Blood *43*. 363–369 (1974).

35 Richman, C. M., Chess, L., and Yankee, R. A.. Purification and characterization of granulocytic progenitor cells (CFU-c) from human peripheral blood using immunologic surface markers. Blood *51* 1–8 (1978).

36 Ruscetti, F W and Chervenick, P Regulation of the release of colony-stimulating activity *from mitogen-stimulated lymphocytes. J Immun. 114* 1513–1517 (1975).

37 Stohlman, F jr Quesenberry P J and Tyler W S The regulation of myelopoiesis as approached with *in vivo* and *in vitro* techniques; in Brown, Progress in hematology vol. VIII, pp. 259–297 (Grune & Stratton, New York 1973)

38 Senner M. A; Bradley T R., Hodgson, G. S., Cline, M. J.; Fry P A., and Sutherland, L.

optimal amount of fresh leukocytes, immobilized in feeder layers, has been established to 1×10^6 cells/ml. A further increase ($\geq 2.0 \times 10^6$ cells) does not enhance stimulation [19] and may actually inhibit colony formation (fig. 3). Such inhibition could result from shortage of nutrients in the medium, or more likely express the negative feedback regulation of myelopoiesis described by *Kurland and Moore* [20, 21]. Underlayers containing $\geq 2.0 \times 10^6$ cryopreserved MNC possessed, however, a high CSA. Further studies are necessary to explain this qualitative difference.

Our results could be used as a basis for developing reference material consisting of large amounts of frozen stimulating cells. Cryopreserved MNC, obtained from a single donor, can thus be used to study alterations of target cells over prolonged periods of time and vice versa.

Acknowledgements

This study was supported by grant FOR.004 AK 71 from the Swiss Cancer League. We are indebted to physicians from the Department of Neurosurgery at our hospital for their kind cooperation and to the numerous patients who readily accepted to donate bone marrow. We thank Dr. *Kurt Deubelbeiss* from our Laboratory for his critical advice during preparation of this manuscript.

References

1 Bettler, M. and Odavic, R.: Some observations on culture of human bone marrow in liquid suspension (in preparation).

2 Böyum, A.: Separation of blood leukocytes, granulocytes and lymphocytes. Tissue Antigens *4*: 269–274 (1974).

3 Bradley, T. R.; Telfer, P. A., and Fry, P.: The effect of erythrocytes on mouse bone marrow colony development *in vitro*. Blood *38*: 353–359 (1971).

4 Brown, C. H. III and Carbone, P. P.: *In vitro* growth of normal and leukemic human bone marrow. J. natn. Cancer Inst. *46*: 989–1000 (1971).

5 Burgess, A. W.; Wilson, E. M. A., and Metcalf, D.: Stimulation by human placental conditioned medium of hemopoietic colony formation by human marrow cells. Blood *49*: 573–583 (1977).

6 Chervenick, P. A. and LoBuglio, A. F.: Human blood monocytes: stimulators of granulocyte and mononuclear colony formation *in vitro*. Science, N.Y. *178*: 164–166 (1972).

7 Chess, L.; Bock, G. N., and Mardiney, M. R.: Restoration of the reactivity of frozen-stored human lymphocytes in the mixed lymphocyte reaction and in response to specific antigens. Transplantation *14*: 728–733 (1972).

8 *Cline, M. J. and Golde, D. W.: Production of* colony-stimulating activity by human lymphocytes. Nature, Lond. *248*: 703–704 (1974).

9 Crowley, J. P.; Rene, A., and Valeri, C. R.: The recovery, structure and function of human blood leukocytes after freeze-preservation. Cryobiology *11*: 395–409 (1974).

10 Dicke, K. A.: Human bone marrow cultures; in van Bekkum and Dicke, *In vitro* culture of hemopoietic cells, pp. 363–366 (Radiobiological Institute, TNO, Rijswijk 1972).

11 Dicke, K. A.; Putten, L. M. van; Oberkleftenburg, V. E., and Lowenberg, B.: Standardization of human bone marrow cultures (Robinson assay) and its application in aplastic anemia; in Metcalf, Condorelli and Peschle, Int. Conf. on Leukemia and Aplastic Anemia, pp. 83–100 (Pensiero Scientifico, Rome 1976).

12 *Van den Engh, G. J.: Quantitative in vitro* studies on stimulation of murine haemopoietic cells by colony stimulating factor. Cell Tissue Kinet. *7*: 537–548 (1974).

13 Golde, D. W.; Finley, T. N., and Cline, M. J.: Production of colony-stimulating factor by human macrophages. Lancet *ii*: 1397–1399 (1972).

14 Golde, D. W. and Cline, M. J.: Identification of the colony-stimulating cell in human peri-

Acta haemat. 61: 251–257 (1979)

Early Increase of Cyclic Adenosine Monophosphate Level Induced by Erythropoietin on Rabbit Bone Marrow Cell Suspensions

F. Chiarini, G. Della Torre, G. Fanò and A. Viti

Istituto di Fisiologia Umana, Università di Perugia, Perugia

Key Words. Erythropoiesis · Erythropoietin · Fluorescein isothiocyanate · Bone marrow · cAMP

Abstract. The effects of an erythropoietin (Ep)-rich plasma fraction and of a commercial preparation of purified Ep (Step I) on the cyclic adenosine monophosphate (cAMP) levels of suspended rabbit bone marrow cells at different incubation times (up to 10 min) were investigated. Both Ep preparations caused an early increase in cAMP concentration. using the plasma fraction a significant increase was found at the 6th, 7th, 9th and 10th min of incubation, whereas the purified Ep produces a significant increase at all incubation times, starting from 1 min. No effect was observed following incubation with fluorescein isothiocyanate (FITC)-inactivated Ep. These findings are discussed and a possible role of cAMP as the intracellular messenger for Ep action is suggested.

Introduction

Although an extensive body of investigation has been devoted to the hormonal regulation of erythropoiesis [9, 13] the detailed mechanisms by which erythropoietin (Ep) induces the erythroid differentiation of the erythropoietin-responsive cells (ERC) of the bone marrow are still incompletely understood. A rapid synthesis of a peculiar mRNA species, as well as the synthesis of some enzymes has been reported to occur in the ERC as early as 15–30 min following Ep administration [4, 8, 16]. Recently evidence has been produced showing that Ep induces the synthesis or the release within the cytoplasm of a specific inductor (marrow cytoplasmic factor: MCF), through the interaction with membrane receptors of ERC [3]. However, the gap between hormone-receptor interaction and MCF appearance remains to be filled.

Various attempts at verifying the possibility that Ep action could be mediated through variations of the intracellular level of cyclic nucleotides (a well-established notion for the action of several proteinaceous hormones [17]) have been reported [5, 10, 12, 18, 21]. Increases of cyclic guanosine monophosphate (cGMP) in rabbit bone

The growth of bone marrow cells in liquid culture. Br J Haemat. 23 221–234 (1972).
39 Vietti, T J., Ragab A. H Gilkerson, E., and Myers, M.. Discordance between the methyl cellulose and agar culture techniques. Expl. Hematol., Copenh. *1* 254 (1973)

Received. May 18, 1978
Accepted. September 5, 1978

Ranko Odavic, Central Hematology Laboratory Inselspital, CH-3010 Berne (Switzerland)

immediately frozen in freon cooled by liquid nitrogen and lyophilized to dryness. The fractions of experiment C were removed from the bath every second minute and processed as those of experiments A and B

AMP Assay Procedure

After determining its dry weight, each sample was denaturated in 5° cold TCA and centrifuged t 27,500 × *g* for 20 min at 4 °C. The supernatant was extracted with ether 5 times and charged on an AG–1 × 8 column (200 400 mesh, Bio-Rad Laboratories, Richmood, V) 3 cm high and 0 3 cm diameter according to *Gilman and Murand* [7]. Satisfactory nucleotide separation was achieved at an elution rate f 2,5 ml/min, with recovery of 80° Determination of cAMP concentrations was carried out with protein-binding method [6]

Data Evaluation

The mean values for each point were compared and the statistical significance of their differences was evaluated by Student t test.

Results

Both Ep-rich plasma fraction and purified Ep induce a significant increase in the concentration of cAMP in our material, as compared to the controls.

Using the Ep-rich plasma fraction (experiment A, see Methods) a significant increase in cAMP concentration (pmol/100 mg dry weight) was first observed 6 min after the addition of the plasma fraction and became more prominent at the 9th and 10th min, reaching a value of 3 pmol/100 mg dry weight as compared to 1 pmol/100 mg dry weight of the controls (fig. 1). During the first 5 min the difference between experimental and control samples was not statistically significant: however a trend towards higher values of cAMP in the treated samples is apparent from the graph of figure 1

A much earlier and more prominent effect was obtained using purified Ep (experiment B see Methods) As shown in the graph of figure 2, 1 min after addition of 0.5 IU of purified Ep the cAMP concentration of the treated samples rose from 1 to 25 pmol/100 mg dry weight and remained at the same value up to the 5th min. A conspicuous decrease of cAMP down to 3 pmol/100 mg dry weight, was observed between the 6th and 8th min, which was followed by a further increase to about



Fig. 1 Effect of Ep-rich plasma fraction on cAMP level of rabbit bone marrow cells. The plasma fraction was added to the experimental samples 5 min after the cell suspensions were prepared (arrow). Dashed line: untreated samples, solid line: experimental samples. Each point represents the mean ± SE, = 10. ℧ = $p < 0.01$ ⊐ = $p < 0.05$.

marrow cultures, in spleen cell suspensions of mice and in fetal liver cells of rats have been found 24 h after treatment with Ep [11] No changes [11] or a decrease [21] of cyclic adenosine monophosphate (cAMP) have been reported to occur in rat fetal liver cells and in splenic cells respectively after Ep stimulation. This lack of effect on cAMP poses puzzling problems, since it stands in sharp contrast to the reported increase in heme synthesis and iron uptake induced by cAMP on ERC [1 5 10]

One reason for the apparent discrepancy between the two sets of data could be traced to species differences as indicated by *Brown and Adamson* [2] As a second possibility the failure to find consistent changes in the level of cAMP [11] could be accounted for by a time factor i.e., one may postulate that the possible Ep-induced variations in cAMP concentrations occur and are completed within the first 15 min, too early to be detected at later times, when mRNA or heme synthesis take place [4]

The present experiments were designed with the aim of verifying the latter hypothesis, namely to ascertain whether a significant change of cAMP could be detected in the first 10 min after Ep stimulation in rabbit bone marrow cells.

Materials and Methods

Rabbit bone marrow cell suspensions were chosen as the experimental material, in view of its recognized sensitivity to cAMP [1, 5] Male animals, 6 months old, fed commercially prepared food (pellets) and given water *ad libitum* were used. Three series of experiments were performed. A, B and C using respectively a fraction of plasma from bled rabbits, prepared according to the technique of *Lowy and Borsook* [14] which is effective in inducing erythropoiesis (experiment A) a commercial preparation of purified Ep (Step I) Connaught Medical Research Lab Toronto) containing 300 IU/500 mg (exp B) a commercial purified Ep inactivated with fluorescein isothiocyanate (FITC) by he method of *Lowy and Borsook* [14] (exp. C).

Animal Procedure

Under sodium pentobarbital anesthesia, abdominal aorta and inferior vena cava were cannulated and an aorta-cava perfusion with warm (38 °C) Hanks' solution was started at a pressure of about 120 mm Hg, to give a flow of approximately 55 ml/min, and maintained until blood had been completely washed out from the hindlimbs. The bone marrow from both femurs was collected in an evacuated flask by means of a teflon catheter inserted through a hole drilled in the distal epiphysis. The marrow collected from each rabbit (approx. 4 ml) was dispersed by gentle stirring.

Incubation Procedure

Experiment A The bone marrow collected from each animal was subdivided into two equal aliquots, one of which was used as a control. The experimental aliquot was dispersed in 8 ml of Hanks solution to which 2 ml of the plasma fraction from bled rabbits [14] was added to a final volume of 12 ml. The control aliquot was dispersed in 10 ml of Hanks solution.

Experiment B The same procedure as in experiment A was used, except that 2 ml of a solution of 0.5 IU/ml purified Ep in Hanks was added in place of the plasma fraction.

Experiment C The marrow collected from each animal was divided into 3 equal aliquots. One of them (control) was dispersed in 6 ml of Hanks' solution 4 ml of Hanks solution + 2 ml of inactivated Ep, namely FITC (4 mg) + Ep (5 IU/ml) and FITC (2 mg) + Ep (5 IU/ml) were added to the second and third aliquots, respectively The above procedures were carried out at 38 °C in a period of 5 min.

The preparations of experiments A and B together with their controls were quickly subdivided into 10 fractions, and incubated in a thermostatic bath at 38 °C for the duration of the experiment. In experiment C the preparation was divided into 6 fractions and similarly incubated. Every single minute from the beginning of the incubation single fractions of A and B preparations together with their controls were removed from the bath,

immediately frozen in freon cooled by liquid nitrogen and lyophilized to dryness. The fractions of periment C were removed from the bath every second minute and processed as those f experiments A and B.

cAMP Assay Procedure

After determining its dry weight, each sample was denaturated in 5° cold TCA and centrifuged at 27,500 × g for 20 min at 4 C. The supernatant as extracted with ether 5 times and charged on an AG-1 × 8 column (200 400 mesh, Bio-Rad Laboratories, Richmond, V) 3 cm high and 0.3 cm diameter according to *Gilman and Murad* [7]. Satisfactory nucleotide separation was achieved at an elution rate of 2.5 ml/min, with recovery of 80%. Determination of cAMP concentrations was carried out with protein-binding method [6].

Data Evaluation

The mean values for each point were compared and the statistical significance of their differences was evaluated by Student' t test.

Results

Both Ep-rich plasma fraction and purified Ep induce a significant increase in the concentration of cAMP in our material, as compared to the controls.

Using the Ep-rich plasma fraction (experiment A see Methods) a significant increase in cAMP concentration (pmol/100 mg dry weight) was first observed 6 min after the addition of the plasma fraction and became more prominent at the 9th and 10th min, reaching a value of 3 pmol/100 mg dry weight as compared to 1 pmol/100 mg dry weight of the controls (fig. 1). During the first 5 min the difference between experimental and control samples was not statistically significant; however a trend towards higher values of cAMP in the treated samples is apparent from the graph of figure 1

A much earlier and more prominent effect was obtained using purified Ep (experiment B see Methods). As shown in the graph of figure 2, 1 min after addition of 0.5 IU of purified Ep the cAMP concentration of the treated samples rose from 1 to 2.5 pmol/100 mg dry weight and remained at the same value up to the 5th min A conspicuous decrease of cAMP down to 3 pmol/100 mg dry weight, was observed between the 6th and 8th min, which was followed by a further increase to about



Fig. 1. Effect of Ep-rich plasma fraction on cAMP level of rabbit bone marrow cells The plasma fraction was added to the experimental samples 5 min after the cell suspensions were prepared (arrow) Dashed line untreated samples, solid line: experimental samples. Each point represents the mean ± SE, = 10. ⊤ = p < 0.01 ◻ = p < 0.05

marrow cultures in spleen cell suspensions of mice and in fetal liver cells of rats have been found 24 h after treatment with Ep [11] No changes [11] or a decrease [21] of cyclic adenosine monophosphate (cAMP) have been reported to occur in rat fetal liver cells and in splenic cells respectively after Ep stimulation. This lack of effect on cAMP poses puzzling problems, since it stands in sharp contrast to the reported increase in heme synthesis and iron uptake induced by cAMP on ERC [1 5 10]

One reason for the apparent discrepancy between the two sets of data could be traced to species differences, as indicated by *Brown and Adamson* [2] As a second possibility the failure to find consistent changes in the level of cAMP [11] could be accounted for by a time factor· i.e. one may postulate that the possible Ep-induced variations in cAMP concentrations occur and are completed within the first 15 min, too early to be detected at later times, when mRNA or heme synthesis take place [4]

The present experiments were designed with the aim of verifying the latter hypothesis, namely to ascertain whether a significant change of cAMP could be detected in the first 10 min after Ep stimulation in rabbit bone marrow cells.

Materials and Methods

Rabbit bone marrow cell suspensions were chosen as the experimental material, in view of its recognized sensitivity to cAMP [1, 5]. Male animals, 6 months old, fed commercially prepared food (pellets) and given water *ad libitum* were used. Three series of experiments were performed. A, B and C using respectively a fraction of plasma from bled rabbits, prepared according to the technique of *Lowy and Borsook* [14] which is effective in inducing erythropoiesis (experiment A)· a commercial preparation of purified Ep (Step I) Connaught Medical Research Lab., Toronto) containing 300 IU/500 mg (exp B) a commercial purified Ep inactivated with fluorescein isothiocyanate (FITC) by he method of *Lowy and Borsook* [14] (exp. C).

Animal Procedure

Under sodium pentobarbital anesthesia, abdominal aorta and inferior vena cava were cannulated and an aorta-cava perfusion with warm (38 °C) Hanks' solution was started at a pressure of about 120 mm Hg, to give a flow of approximately 55 ml/min, and maintained until blood had been completely washed out from the hindlimbs. The bone marrow from both femurs was collected in an evacuated flask by means of a teflon catheter inserted through a hole drilled in the distal epiphysis. The marrow collected from each rabbit (approx. 4 ml) was dispersed by gentle stirring.

Incubation Procedure

Experiment A The bone marrow collected from each animal was subdivided into two equal aliquots, one of which was used as a control. The experimental aliquot was dispersed in 8 ml of Hanks' solution to which 2 ml of the plasma fraction from bled rabbits [14] was added to a final volume of 12 ml. The control aliquot was dispersed in 10 ml of Hanks' solution.

Experiment B The same procedure as in experiment A was used, except that 2 ml of a solution of 0.5 IU/ml purified Ep in Hanks was added in place of the plasma fraction.

Experiment C The marrow collected from each animal was divided into 3 equal aliquots. One of them (control) was dispersed in 6 ml of Hanks' solution· 4 ml of Hanks' solution + 2 ml of inactivated Ep, namely FITC (4 mg) + Ep (5 IU/ml) and FITC (2 mg) + Ep (5 IU/ml) were added to the second and third aliquots, respectively The above procedures were carried out at 38 C in a period of 5 min.

The preparations of experiments A and B together with their controls were quickly subdivided into 10 fractions, and incubated in a thermostatic bath at 38 °C for the duration of the experiment. In experiment C the preparation was divided into 6 fractions and similarly incubated. Every single minute from the beginning of the incubation single fractions of A and B preparations together with their controls were removed from the bath.

gated in another series of experiments (experiment C see Methods) in which the effects of Hanks solution were compared with those induced by two doses 10 times higher of purified Ep (5 IU), previously inactivated with 2 and 4 mg of FITC, respectively As shown in figure 3 the small, aspecific increase in cAMP was identical in magnitude and time course in the preparations treated with Hanks solution alone and 5 IU Ep + 2 mg FITC, whereas a more prominent increase was observed in those treated with the same amount of Ep, inactivated by 4 mg FITC. Also in this experiment, however the cAMP increase subsided to control levels within 2 min.

Discussion

The results of our investigation show that an increase in cAMP concentration is induced in rabbit bone marrow cells following Ep administration. The hypothesis that this increase may be the aspecific result of the experimental treatments could be advanced on the basis of the slight and transient increase observed in the untreated controls. This possibility is, however difficult to reconcile with the striking differences in both magnitude and time course of the two effects and is ruled out by the finding that the increase induced by a 10 times higher dose of Ep inactivated with 2 mg FITC is identical to that observed in the untreated controls.

One is led, therefore to the conclusion that the increase in cAMP concentration observed after Ep administration is specific in nature, clearly distinguished from that aspecifically induced by the experimental manipulations. The significantly higher levels of cAMP observed following treatment with the same dose of Ep inactivated with 4 mg FITC can thus be interpreted as the result of the aspecific action of the excess FITC, also in view of the fact that the increase runs the same time course as that of the controls.

In our material the effects of purified Ep and of the Ep-rich plasma fraction are consistent, in that both induce a specific increase in cAMP concentration. They differ markedly however in magnitude and time course, a significant increase being observed 1 min after the addition of purified Ep, whereas the first significant increase appears 6 min after addition of the Ep-rich plasma fraction. The reasons for this discrepancy are not easily understood. Although the Ep content of the plasma fraction we have used has not been determined, the possibility that the observed differences could be attributed, in part at least, to dose differences appears as a likely one, since in this case both the smallness of the increase, and the variability inherent in the determinations would concur in obscuring the true time course of the effect. Obviously direct evidence is needed to clarify this point.

The increase in cAMP concentration induced by purified Ep shows two characteristic features, namely a fast rise within 1 min and a decline between the 5th and 9th min of incubation, followed by a further rise to high values. The rapidity in onset we have observed is in agreement with the times of hormonal actions mediated by cAMP as the intracellular messenger [4 8, 13 16] On the other hand, the apparent discrepancy between our results and the negative ones reported by *Graber et al* [11] could be accounted for by the fact that these authors determined the levels of cyclic nucleotides

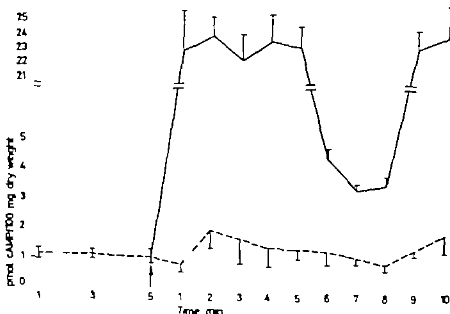


Fig. 2. Effect of purified EP (Step 1) on cAMP level of rabbit bone marrow cells. Ep was added to the experimental samples (0.5 IU/ml) 5 min after the cell suspension was prepared (arrow). Dashed line: untreated samples; solid line: experimental samples. Each point represents the mean ±SE, n = 6. All differences between control and experimental points are highly significant ($p < 0.001$)

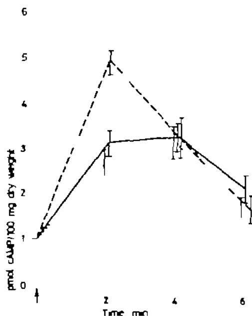


Fig. 3. Comparison between cAMP levels of untreated samples (solid line) and of samples treated with Ep (5 IU/ml) inactivated with 2 mg FITC (dotted line) and with 4 mg FITC (dashed line), added at zero time (arrow) during the first 6 min of incubation. Each point represents the mean ± SE, n = 4, □ = $p < 0.01$

23 pmol at the 9th and 10th min. The differences between experimental and control values were all *statistically significant.*

A consistent finding of our experiments was the transient increase in cAMP concentrations which occurred in the controls of both A and B experiments 2 min after the addition of Hanks solution (fig. 1, 2, dashed lines). This increase, albeit consistent, was not prominent and subsided within 1 or 2 min, thus suggesting the possibility of a nonspecific stimulation of cAMP synthesis.

The specific nature of the cAMP increases observed after addition of Ep was investi-

gated in another series of experiments (experiment C, see Methods), in which the effects of Hanks solution were compared with those induced by two doses 10 times higher of purified Ep (5 IU), previously inactivated with 2 and 4 mg of FITC, respectively. As shown in figure 3 the small, aspecific increase in cAMP was identical in magnitude and time course in the preparations treated with Hanks solution alone and 5 IU Ep + 2 mg FITC, whereas a more prominent increase was observed in those treated with the same amount of Ep, inactivated by 4 mg FITC. Also in this experiment, however the cAMP increase subsided to control levels within 2 min.

Discussion

The results of our investigation show that an increase in cAMP concentration is induced in rabbit bone marrow cells following Ep administration. The hypothesis that this increase may be the aspecific result of the experimental treatments could be advanced, on the basis of the slight and transient increase observed in the untreated controls. This possibility is, however difficult to reconcile with the striking differences in both magnitude and time course of the two effects and is ruled out by the finding that the increase induced by a 10 times higher dose of Ep inactivated with 2 mg FITC is identical to that observed in the untreated controls.

One is led therefore, to the conclusion that the increase in cAMP concentration observed after Ep administration is specific in nature, clearly distinguished from that aspecifically induced by the experimental manipulations. The significantly higher levels of cAMP observed following treatment with the same dose of Ep inactivated with 4 mg FITC can thus be interpreted as the result of the aspecific action of the excess FITC, also in view of the fact that the increase runs the same time course as that of the controls.

In our material the effects of purified Ep and of the Ep-rich plasma fraction are consistent, in that both induce a specific increase in cAMP concentration. They differ markedly however in magnitude and time course, a significant increase being observed 1 min after the addition of purified Ep whereas the first significant increase appears 6 min after addition of the Ep-rich plasma fraction. The reasons for this discrepancy are not easily understood. Although the Ep content of the plasma fraction we have used has not been determined, the possibility that the observed differences could be attributed, in part at least, to dose differences appears as a likely one, since in this case both the smallness of the increase and the variability inherent in the determinations would concur in obscuring the true time course of the effect. Obviously direct evidence is needed to clarify this point.

The increase in cAMP concentration induced by purified Ep shows two characteristic features, namely a fast rise within 1 min and a decline between the 5th and 9th min of incubation, followed by a further rise to high values. The rapidity in onset we have observed is in agreement with the times of hormonal actions mediated by cAMP as the intracellular messenger [4–8, 13–16]. On the other hand, the apparent discrepancy between our results and the negative ones reported by *Graber et al.* [11] could be accounted for by the fact that these authors determined the levels of cyclic nucleotides

15 min, 30 min and hours after the start of incubation with Ep therefore missing the early increase we have observed Along the same line of reasoning, the decline in the level of cAMP down to values only slightly higher than those of the control, which takes place from the 5th to 9th incubation minutes may explain the lack of the effect found by *Graber et al* [11] at the 5th min of incubation

The nature of the diphasic increase in cAMP of bone marrow cells following Ep stimulation is unclear at present. It could be speculated that it may reflect the operation of an intracellular regulatory system on cyclic nucleotide levels alternatively the two peaks we have found could be interpreted as the expression of two temporally separated sets of events, elicited both by Ep stimulation and involving an increase of cAMP Clearly further evidence is needed to decide between these possibilities in particular the temporal relationships between the changes of cAMP and cGMP following stimulation with different doses of Ep has to be established, a work which is presently being carried out.

Taken together our findings suggest that cAMP may play a role as intracellular messenger in the response of hematopoietic cells to Ep stimulation a mechanism proposed by several investigators [1 5 10] but still lacking universal acceptance [11 18 21] This suggestion can be validated only if a causal relationship between the Ep-induced cAMP increase and the physiological actions of Ep on bone marrow cell will be demonstrated In particular the possibilities that cAMP may be involved in Hb synthesis, marrow cell proliferation or both, need to be explored. It is worth mentioning in this context that a role of cAMP in cell proliferation has been proposed [19] and that there is some evidence that a transient increase in cAMP is required both as a mitogenic signal [15] and to drive lymphocytes from the G_1 to S phase of the mitotic cycle upon mitogenic stimulation [20] Experiments aimed to this end are in progress.

Acknowledgements

The authors wish to thank Prof. *Franco Magni* for advice and encouragement throughout this work. The technical assistance of Mr *G Laguardia* is gratefully acknowledged.

References

1 Bottomley S. S Whitcomb W H., Smithet, G A., and Moore, M. Z. Effect of cyclic adenosine 3 5-monophosphate on bone marrow Δ-aminolevulinic acid synthetase and erythrocyte iron uptake. J Lab clin. Med. 77 793–801 (1971).

2 Brown, J E. and Adamson, J W Studies of the influence of cyclic nucleotides on in vitro haemoglobin synthesis. Br J Haemat. 35 193–207 (1977)

3 Chang, S C. S. Sikkema, D and Goldwasser E. Evidence for an erythropoietin receptor protein on rat bone marrow cells. Biochem. biophys. Res. Commun. 57 399–405 (1974).

4 Dyaldetti, M., Preisler H Marks, P A and Rifkind, R. A.. Erythropoietin effects on fetal mouse erythroid cells. II. Nucleic acid synthesis and the erythropoietin-sensitive cell J biol. Chem. 247 731–735 (1971)

5 Dukes, P P Potentiation of erythropoietin effects in marrow cell cultures by prostaglandin E or cyclic 3 5 AMP Blood 38 822 (1971)

6 Gilman, G A A protein binding assay for adenosine 3 5 cyclic monophosphate. Proc. natn. Acad. Sci. USA 67 305–312 (1970).

7 Gilman, G A. and Murad, F Assay of cyclic nucleotides by receptor protein binding displacement. Meth. Enzym. 38 49–51 (1974).

8 Goldwasser E. and Gross, M On the mechanism of erythropoietin induced differentiation. IX. Induced synthesis of 9S ribonucleic acid and hemoglobin. J biol. Chem. 246 2480–2486 (1971).

9 Gordon, A. S. Erythropoietin hormonal effect; in Peptide hormones, p. 1103 (North-Holland, Limerick 1973).

10 Gorshein, D Reisner E. D and Gardner F H. Tissue culture of bone marrow V Effect of 5 B (H) steroids and cyclic AMP on heme synthesis. Am. J Physiol. 28 1024–1028 (1975)

11 Graber S. E., Bomboy J D., Salmon, W D and Krantz, S. B Effect of erythropoietin preparations on cyclic AMP and cyclic GMP levels in rat fetal liver cell cultures J Lab. clin. Med. 90. 162 170 (1977).

12 Graber S E Carrillo, M and Krantz, S. B Lack of effect of erythropoietin on 3 5' AMPc levels in rat fetal liver cells. J Lab clin. Med. 83 288–295 (1974).

13 Krantz, S. B Recent contributions to the mechanism of action and clinical relevance of erythropoietin. J Lab clin. Med. 82 847–857 (1973)

14 Lowy P H. and Borsook, H.. Preparations and properties of erythropoietin concentrates from rabbit plasma and human urine in Erythropoiesis, p. 32 (Grune & Stratton, New York 1962)

15 Parker C W Cyclic AMP and the immune response, in Advances in cyclic nucleotide research, vol 4, p 2 (Raven Press, New York 1974).

16 Piantadosi, C. A., Dickerman, H. W and Spivak, J L. Sequential activation of splenic nuclear RNA polymerases by erythropoietin. J clin. Invest. 57 20–26 (1976).

17 Robison, G A., Butcher R. W and Sutherland, E. W Cyclic AMP (Academic Press, New York 1971).

18 Rodgers, G. M., Fisher J W and George, W J Elevated cyclic GMP concentrations in rabbit bone marrow culture and mouse spleen following erythropoietic stimulation. Biochem. biophys. Res. Commun. 70 287–294 (1976).

19 Wang, T Schepperd, R., and Foker J E Rise and fall of cyclic AMP required for onset of lymphocyte DNA synthesis. Science, N Y 201 155–157 (1978).

20 Whitfield, J F MacManus, G P Rixon, R. H., Boynton, A. L., Yondale, T and Swierenga, S Positive control of cell proliferation by interplay of calcium ions and nucleotides. In Vitro 12. 1 18 (1976).

21 Winkert, J Depression of splenic cyclic AMP levels by erythropoietic stimuli. Fed. Proc. Fed Am. Socs exp. Biol. 32 535 (1973).

Received: June 22, 1977
Accepted. November 8, 1978

F Chiolni, Istituto di Fisiologia Umana
dell'Università di Perugia,
via del Giochetto,
I-06100 Perugia (Italy)

Acta haemat. *61* 258–263 (1979)

Erythroid Repopulating Ability of Bone Marrow Cells in Polycythaemic Mice

P. Milenković and V. Pavlović Kentera

Institute for Medical Research, Beograd

Key Words. Erythroid repopulating ability · Stem cells · Polycythaemia

Abstract. The erythroid repopulating ability (ERA) of femoral bone marrow cells was determined in polycythaemic CBA, $C_{57}Bl$ and Balb/ mice. Polycythaemia was induced by hypertransfusion or exposure to chronic hypoxia in silicone rubber membrane enclosures. The results obtained demonstrate decreased ERA after both types of polycythaemia, with the exception of ex hypoxic Balb/ mice. A decrease in the number of erythroid repopulating cells in DNA synthesis was found in hypertransfused and exhypoxic CBA mice in comparison to normal values. These studies indirectly indicate the contribution of cells committed to erythropoiesis in measurement of ERA.

The existence of an erythroid committed precursor (ECP) compartment seems to be well established. Different *in vivo* and *in vitro* techniques may be used to study this heterogenous population of cells [1, 11, 19, 22]. One of the functional *in vivo* tests, namely the erythroid repopulating ability (ERA) of bone marrow cells may measure a part of the ECP compartment, in addition to common stem cells [3, 4, 6, 7, 15].

Induction of polycythaemia leads to the suppression of erythropoiesis, with no recognisable erythroid cells in the bone marrow. By means of *in vitro* techniques, changes in the ECP compartment without corresponding changes in the number of femoral pluripotent haemopoietic stem cells (CFU_s) were demonstrated to appear in polycythaemic mice [5, 9, 23]. However *in vitro* measurement of ECP, although useful for estimation of the number of particular cells, does not provide sufficient information on the functional capabilities of these cells.

In this work the ERA of bone marrow cells in polycythaemic mice was determined in order to see whether induction of polycythaemia influences the repopulating capacity of bone marrow cells as could be expected from the changes in ECP after induction of polycythaemia.

Materials and Methods

The experiments were performed on female mice of CBA/H, $C_{57}Bl$ and Balb/ strains.

Polycythaemia was induced by two injections of washed syngeneic red blood cells (*hypertransfusion induced polycythaemia*) or by Lange method [12], in which mice were exposed to 50 mm Hg pO_2 in silicone rubber membrane enclosures, as modified by *Ichiki and Lange* [8] for 3 weeks ($C_{57}Bl$, Balb) and 2 weeks (CBA) respectively (*exhypoxic polycythaemia*).

ERA of femoral bone marrow cells was determined as described by *Tweatyman and Blackett* [22] 7 days after grafting femoral bone marrow cells to the irradiated recipient mice. The 24 h uptake of ^{59}F into peripheral blood was measured after injection of 0.5 μCi radio-iron.

The peripheral blood ^{59}Fe incorporation obtained after injection of 5×10^4 to 15×10^4 bone marrow cells was calculated per 10^4 injected bone marrow cells and converted to the incorporation equivalent of the injection of bone marrow cells from one femur [2]. The results for ERA/femur in polycythaemic animals were compared to the ERA/femur of normal mice of the same strain. The significance of differences between ERA in normal and polycythaemic animals was tested using Student t test.

The number of animals used as donors of femoral bone marrow cells for ERA measurement are given in table IV as well as the corresponding mean haematocrit values.

In *hypertransfused mice* ERA was determined (1) in CBA mice on the 7th, 8th and 9th day after hypertransfusion. Since no difference was noted between those groups, the obtained results are presented together: (2) in $C_{57}Bl$ mice on the 4th, 5th and 6th day after hypertransfusion. Since no difference between those groups was found, the obtained results are presented together. (3) In Balb/c mice on the 5th day after hypertransfusion. I *ex-hypoxic mice* ERA was determined: (4) in CBA and Balb mice on the 7th and 8th day after termination of hypoxia. (5) in $C_{57}Bl$ mice on the 7th and 8th day after termination of hypoxia. Since no difference was found between those groups, the results obtained are presented together.

Spleen colony assay (CFU_s) The number of CFU was measured using the method of *Till and McCulloch* [20]. A convenient number of femoral bone marrow cells was injected intravenously into 1,000 rad irradiated recipient CBA mice. The recipient animals were sacrificed 8 days later the removed spleens fixed in Bouin solution and surface colonies counted. The CFU number was calculated per bone marrow cells of one femur.

Cytosine arabinoside (AraC) suicide A single dose of 200 mg/kg AraC (Alexan, Lek) was injected intravenously into donor mouse. 2 h later femoral bone marrow cells were injected into irradiated recipient animals and the CFU and ERA measured, as already described in this text.

By analogy to the determination of CFU and ERC 'suicide' with ^{3}H-thymidine *in vivo* [11] all the results for AraC 'suicide' of erythroid repopulating cells and CFU were expressed as per cent kill in the AraC treated group. The results for CFU_s/femur and ERA/femur obtained after AraC injection were compared to the results obtained in the animals not treated with AraC. The percent CFU and ERA killed were then calculated in AraC-treated groups and the mean percent of CFU and ERA killed determined. The standard error of the mean was obtained using the equation for standard error of CFU or ERA difference in AraC-treated and not treated groups. This equation was originally used by *Lord et al.* [13] for determination of the standard error for CFU ^{3}H-thymidine 'suicide' *in vitro*.

Results

In CBA $C_{57}Bl$ and Balb/c mice with hypertransfusion-induced polycythaemia, ERA of femoral bone marrow cells was lower as compared to normal mice (table I). The same was true for ERA measured in exhypoxic polycythaemic CBA and $C_{57}Bl$ mice but not in Balb mice. As shown in table I the difference between ERA of bone marrow cells of normal and polycythaemic mice with both hypertransfusion and exhypoxic polycythaemia was statistically significant, with the exception of Balb mice. This is a strain of mice in which polycythaemia cannot be induced by hypoxia [16]. There was no difference between ERA values in $C_{57}Bl$ mice with exhypoxic polycy-

Acta haemat. 61 258–263 (1979)

Erythroid Repopulating Ability of Bone Marrow Cells in Polycythaemic Mice

P Milenković and V Pavlović Kentera
Institute for Medical Research, Beograd

Key Words. Erythroid repopulating ability Stem cells Polycythaemia

Abstract. The erythroid repopulating ability (ERA) of femoral bone marrow cells was determined in polycythaemic CBA C_{57}Bl and Balb/ mice. Polycythaemia was induced by hypertransfusion or exposure to chronic hypoxia in silicone rubber membrane enclosures. The results obtained demonstrate decreased ERA after both types of polycythaemia, with the exception of ex hypoxic Balb/ mice A decrease in the number of erythroid repopulating cells in DNA synthesis was found in hypertransfused and exhypoxic CBA mice in comparison to normal values. These studies indirectly indicate the contribution of cells committed to erythropoiesis in measurement of ERA.

The existence of an erythroid committed precursor (ECP) compartment seems to be well established. Different *in vivo* and *in vitro* techniques may be used to study this heterogenous population of cells (1 11 19 22] One of the functional *in vivo* tests, namely the erythroid repopulating ability (ERA) of bone marrow cells may measure a part of the ECP compartment, in addition to common stem cells [3 4 6 7 15]

Induction of polycythaemia leads to the suppression of erythropoiesis, with no recognisable erythroid cells in the bone marrow By means of *in vitro* techniques, changes in the ECP compartment without corresponding changes in the number of femoral pluripotent haemopoietic stem cells (CFU_s) were demonstrated to appear in polycythaemic mice [5 9 23] However *in vitro* measurement of ECP although useful for estimation of the number of particular cells, does not provide sufficient information on the functional capabilities of these cells.

In this work the ERA of bone marrow cells in polycythaemic mice was determined in order to see whether induction of polycythaemia influences the repopulating capacity of bone marrow cells as could be expected from the changes in ECP after induction of polycythaemia.

Materials and Methods

The xperiments were performed on female mice of CBA/H, C_{57}Bl and Balb/ strains.

Table III. Cytosine arabinoside 'suicide' of erythroid repopulating ERA cells and CFU$_s$ in normal and polycythaemic CBA mice (number of recipient animals in parentheses) percent of cells killed after 200 mg/kg cytosine arabinoside intravenously in h (mean ± SE), standard errors calculated according to *Lord et al* [13]

	Normal	Polycythaemic		
		Day after hypertransfusion		7th day after hypoxia
		7	8	
CFU$_s$	8 2 ± 4 7 (10)	6 4 ± 8.1 (7)		
ERA cells	65 ± 5 6 (10)	24 ± 17.3 (9)	20 ± 18 6 (7)	21 ± 6 8 (10)

Table IV. Level of polycythaemia in hypertransfused and exhypoxic mice used as donors for ERA measurement (mean ± SE) number of animals used as recipients of bone marrow cells for ERA measurement in parentheses

	Hypertransfusion		Hypoxia	
	number of animals	PCV %	number of animals	PCV %
CBA	15 (38)	59 ± 0 67	5 (9)	72 ± 1 0
C57Bl	31 (49)	63 ± 0 72	26 (39)	70 ± 0 65
Balb/	6 (7)	81 ± 1 10	5 (16)	56 ± 2 30

bilities of these cells and therefore the ERA measurement in polycythaemic mice done in this work was expected to provide indirect evidence for the degree of ECP contribution in this test.

The decrease of ERA in polycythaemic mice demonstrated in this work was not due to the change in the number or proliferation rate of CFU The number of femoral CFU *from day 5 to 10 after hypertransfusion* was in the range that could be found in normal animals, as can be seen from table II At the same time CFU cytosine arabinoside 'suicide demonstrated an unchanged proliferation rate of CFU in polycythaemic mice (table III) In polycythaemic mice the same number of femoral CFU as in normal animals has also been found by other authors [10, 18, 21 23] However there is a possibility that CFU in bone marrow cells of polycythaemic mice injected for ERA measurement might have lower seeding efficiency than in normal mice, which could influence ERA measurement. Unfortunately a technique for determination *of seeding efficiency of erythroid repopulating* cells has not been described and a possible influence of decreased CFU seeding on ERA measurements cannot be excluded.

Another explanation for the decreased ERA found in polycythaemic mice that should be considered is the change in population size of ECP In this work we ha e not measured ERA and ECP concomitantly and therefore we can discuss our results only in conjunction with the results of ECP measurements obtained in polycythaemic mice by other authors.

On the basis of different damage caused by several cytotoxic drugs to ERC and erythroid repopulating cells, *Millar et al.* [15] postulated that erythroid committed cells that had acquired the ability to respond to erythropoietin lost the ability to contribute to ERA. Differences in damage to ERC and erythroid repopulating cells could be explained as a consequence of the different degree of survival of these cells after cytotoxic drugs, which reduce their population

thaemia in comparison to mice with hypertransfusion polycythaemia. However in CBA mice although ERA values were lower in animals with both types of polycythaemia than in normal mice the decrease was more pronounced in hypertransfusion than in polycythaemia induced by hypoxia, the difference being statistically significant ($p < 0.01$)

ERA suicide was lower in CBA mice with both types of polycythaemia (table III) as compared to the ERA 'suicide obtained in normal mice. On the contrary in the CFU compartment, changes were not noted. The relative number of CFU in the femur of hypertransfused CBA mice was within the range of values found in animals although some fluctuations were noted (table II). CFU suicide was changed in hypertransfused as compared normal animals (table III)

Table I *ERA/femur in polycythaemic mice of three different strains presented as percent of ERA/femur in normal mice of the same strain (mean ± SE)*

	Polycythaemia induced by	
	hypertransfusion	hypoxia
CBA	41 ± 2.43	7? ± 4.10*
CaBl	38 ± 3.16	67 ± 3.90*
Balb/	35 ± 4.77*	80 ± 10.46 NS

Statistical significance of differences between ERA/femur in polycythaemic mice and ERA/femur in normal mice was tested by Student's t test. NS = Not significant. p < 0.05. p < 0.01

Discussion

The presented results demonstrate suppression of erythropoiesis has a definitive decreasing effect on ERA. On the basis of ERA measurement after cytotoxic drugs *Blackett* and co-workers [3, 14, 15] suggested that this test might depend on early cells committed to erythropoiesis in addition to CFU_e. The possible dependence of repopulating tests on committed cells was also suggested by *Hellman* and co-workers [4, 6, 7]. Recent experiments in our laboratory (unpubl. observations) demonstrate fast ERA recovery that precedes CFU regeneration in polycythaemic mice treated with cyclophosphamide (Cy). Since the erythropoietin responsive cells (ERC) remained low at the time when ERA had recovered to 80% control it was concluded that in polycythaemic mice treated with Cy the ERA test measures not only the CFU but also early committed cells to erythropoiesis.

However the number of CFU_s and ECP might not reflect the functional capa-

Table II CFU_s/femur in normal and hypertransfused CBA mice (number of recipient animals in parentheses) mean ± SE

Normal	Polycythaemic day after hypertransfusion			
	5	7	8	10
3,321 ± 154 (49)	3,865 ± 139 (21)	3,107 ± 226 (7)	5,354 ± 310 (7)	3,415 ± 154 (10)

Table III. Cytosine arabinoside suicide of erythroid repopulating ERA cells and CFU in normal and polycythaemic CBA mice (number of recipient animals in parentheses) percent of cells killed after 200 mg/kg cytosine arabinoside intravenously *in vivo* (mean ± SE), standard errors calculated according to *Lord et al.* [13]

	Normal	Polycythaemic		
		Day after hypertransfusion		7th day after hypoxia
		7	8	
CFU	8.2 ± 4.7 (10)	6.4 ± 8.1 (7)		
ERA cells	65 ± 5.6 (10)	24 ± 17.3 (9)	20 ± 18.6 (7)	21 ± 6.8 (10)

Table IV. Level of polycythaemia in hypertransfused and exhypoxic mice used as donors for ERA measurement (mean ± SE) number of animals used as recipients of bone marrow cells for ERA measurement in parentheses

	Hypertransfusion		Hypoxia	
	number of animals	PCV %	number of animals	PCV %
CBA	15 (38)	59 ± 0.67	5 (9)	77 ± 1.0
C57Bl	31 (49)	63 ± 0.72	26 (39)	70 ± 0.65
Balb	6 (7)	81 ± 1.10	5 (16)	56 ± 2.30

bilities of these cells and therefore the ERA measurement in polycythaemic mice done in this work was expected to provide indirect evidence for the degree of ECP contribution in this test.

The decrease of ERA in polycythaemic mice demonstrated in this work was not due to the change in the number or proliferation rate of CFU. The number of femoral CFU from day 5 to 10 after hypertransfusion was in the range that could be found in normal animals, as can be seen from table II. At the same time CFU cytosine arabinoside suicide demonstrated an unchanged proliferation rate of CFU in polycythaemic mice (table III). In polycythaemic mice the same number of femoral CFU as in normal animals has also been found by other authors [10–18, 21–23]. However there is a possibility that CFU in bone marrow cells of polycythaemic mice injected for ERA measurement might have lower seeding efficiency than in normal mice which could influence ERA measurement. Unfortunately a technique for determination of seeding efficiency of erythroid repopulating cells has not been described and a possible influence of decreased CFU seeding on ERA measurements cannot be excluded.

Another explanation for the decreased ERA found in polycythaemic mice that should be considered is the change in population size of ECP. In this work we have not measured ERA and ECP concomitantly and therefore we can discuss our results only in conjunction with the results of ECP measurements obtained in polycythaemic mice by other authors.

On the basis of different damage caused by several cytotoxic drugs to ERC and erythroid repopulating cells, *Millar et al.* [15] postulated that erythroid committed cells that had acquired the ability to respond to erythropoietin lost the ability to contribute to ERA. Differences in damage to ERC and erythroid repopulating cells could be explained as a consequence of the different degree of survival of these cells after cytotoxic drugs, which reduce their population

size differently However reduction of ERC population size has been found in polycythaemic mice not treated with cytotoxic drugs [17] although production of ERC exists even in long term polycythaemia [10 24] It is therefore possible that reduction in the population size of ERC, probably caused by decreased amplification from early committed precursors, could contribute to the decrease of ERA in polycythaemic mice observed in this work. In addition, high suicide of erythroid repopulating cells in normal animals was found in this work which was in the range of values for ERC suicide *in vivo* [11] However suicide of erythroid repopulating cells decreased after induction of polycythaemia which could only be explained on the basis of functional changes that occur in ERA in the absence of a demand for differentiated cells.

It could be suggested that although a decrease of ERA and suicide of erythroid repopulating cells was demonstrated in polycythaemic mice, these changes should be further elucidated The change of ERA, observed in polycythaemic mice in this work, could be considered to demonstrate the capability of this test to detect functional changes within the erythroid stem cell compartment

Acknowledgement

This work was supported by a grant from the Medical Scientific Fund of Serbia. Animals were irradiated in the Department of Radiotherapy Radiological Institut Medical School, Beograd

References

1 Axelrad, A. A McLeod, D L. Shreeve, N M and Heatch, D S.. Properties of cells that produce erythroid colonies *in vitro* in Robinson, Hemopoesis in culture, pp. 226–234 (US Government Printing Office, Washington 1974).

2 Blackett, N M and Adams, K. Cell proliferation and the action of cytotoxic agents on haemopoietic tissue. Br J Haemat. *23* 751–758 (1972)

3 Constable, T B and Blackett, N M. Comparison of effect of four cytotoxic agents on granulocytic and erythroid repopulating ability of rat bone marrow J natn. Cancer Inst. *48*. 941–948 (1972)

4 Goodman R., Grate, H. E., Hannon, E., and Hellman, S. Hematopoietic stem cells: effect of preirradiation, bleeding and erythropoietin on thrombopoietic differentiation. Blood *49* 253–261 (1977).

5 Gregory G J., McCulloch, E. A., and Till, J E.. Erythropoietic progenitors capable of colony formation in culture: state of differentiation. J cell Physiol. *81* 411–420 (1973)

6 Hellman, S., Grate, H. E and Chaffey J T Effects of radiation on the stem cell compartment to differentiate into granulocytic and erythrocytic progeny Blood *34* 141–156 (1969).

7 Hellman, S. Grate, H. E. Chaffey J T and Carmel, R.. Hematopoietic stem cell compartment pattern of differentiation following radiation or cyclophosphamide; in Stohlman, Hemopoietic cellular proliferation, pp 36–45 (Grune & Stratton, New York 1970)

8 Ichiki, A. T and Lange, R. D A method for the concentration of erythropoietin from human urine Biochem. Med. *10* 50–60 (1974)

9 Iscove, N N The role of erythropoietin in regulation of population size and cell cycling of early and late erythroid precursors in mouse and bone marrow Cell Tiss Kinet. *10* 323–334 (1977).

10 Kubanek, B. Bock, O Heit, W Bock, E., and Harris, E. B. Size and proliferation of stem cell compartments in mice after depression of erythropoiesis in Hemopoietic Stem Cells, Ciba Foundation Symposium, pp. 43–55 (Elsevier Amsterdam 1973)

11 Lajtha, L. G Pozzi, L. V., Schofield, R., and Fox, M. Kinetic properties of haemopoietic stem cells. Cell Tiss. Kinet. 2. 39–49 (1969).

12 Lange, R. D Simmonds, M L., and Dibelius, N R.. Polycythaemic mice produced by hy

poxia in silicone rubber membrane enclosures: a new technique. Proc. Soc. exp. Biol. Med *122*, 761 764 (1966).

13 Lord, B J Lajtha, L. G and Gidali, J Measurement of kinetic status of bone marrow pre cursor cells: three cautionary tales. Cell Tiss. Kinet 7 507–515 (1974)

14 Millar L J and Blackett, N. M The effect of various cytotoxic agents on the erythroid pre cursors in rat bone marrow Br J Haemat 26. 535–541 (1974).

15 Millar J L Constable, T B., and Blackett, N M Delayed response to erythropoietin in polycythaemic animals treated with cytotoxic agents Cell Tiss. Kinet. 7 363–370 (1974)

16 Nohr M L. Inefficiency of erythropoietic re sponse of Balb/c mice t hypo ia. Am. J Phy siol. *213* 1285–1289 (1967).

17 O'Kunewick, J P and F lton, D Comparison of erythropoietin response in mice following polycythaemia produced by transfusion or hy poxia Blood 36 239–245 (1970)

18 Shadduck, R. K. Tyler W S., Porcellini, A Howard, D E., and Stohlman, F jr Stem cell response to alternative suppression and stimulation of the erythroid system. Radiat. Res 50 379–387 (1972).

19 Stephenson, J R., Axelrad, A. A., McLeod, D L., and Shreeve, N M Induction f colonies of hemoglobin synthetizing cells by erythropoietin in vitro. Proc. natn. Acad. Sci USA 68, 1542 1546 (1971).

20 Till, J E and McCulloch, E. A. A direct measurement of radiation sensitivity of normal mouse bone marrow cells. Radiat. Res. *14* 213–222 (1961).

21 Till, J E., Siminovitch, L., and McCulloch, E. A. The effect of plethora on growth and dif ferentiation of normal hemopoietic colony forming cells transplanted into mice of genotype W/Wᵛ Blood 29 102 113 (1967).

22 Tweentyman P R. and Blackett, N. M Action of cytotoxic agents on erythroid system of the mouse. J natn. Cancer Inst. *44* 117 123 (1970).

23 Wagemaker G Ober Kieftenburg, V E., Brouwer A., and Petersbough, M. F Some characteristics of *in vitro* erythroid colony and burst-forming units. In Baum and Ledney E perimental hematology today pp. 103–110 (Springer New York 1977).

24 Wangenheim, H. R. von; Schofield, R., Kyffin, S., and Klein, B. Studies of erythroid-committed precursor cell in polycythaemic mice Biomedicine 27 337–340 (1977).

Received October 8, 1978
Accepted. November 17 1978

Dr P Milenković,
Institut za medicinska istraživanja,
Bulevar JNA 10,
PO Box 721 11001 Beograd (Yugoslavia)

size differently However reduction of ERC population size has been found in polycythaemic mice not treated with cytotoxic drugs [17] although production of ERC exists even in long-term polycythaemia [10 24] It is therefore possible that reduction in the population size of ERC, probably caused by decreased amplification from early committed precursors, could contribute to the decrease of ERA in polycythaemic mice observed in this work. In addition high suicide of erythroid repopulating cells in normal animals was found in this work which was in the range of values for ERC suicide *in vivo* [11] However suicide of erythroid repopulating cells decreased after induction of polycythaemia which could only be explained on the basis of functional changes that occur in ERA in the absence of a demand for differentiated cells.

It could be suggested that although a decrease of ERA and suicide of erythroid repopulating cells was demonstrated in polycythaemic mice these changes should be further elucidated The change of ERA, observed in polycythaemic mice in this work, could be considered to demonstrate the capability of this test to detect functional changes within the erythroid stem cell compartment.

Acknowledgement

This work was supported by a grant from the Medical Scientific Fund of Serbia. Animals were irradiated in the Department of Radiotherapy Radiological Institute Medical School, Beograd

References

1 Axelrad, A A. McLeod, D L Shreeve, N M., and Heatch, D S. Properties of cells that produce erythroid colonies *in vitro* In Robinson, Hemopoiesis in culture, pp 226–234 (US Government Printing Office, Washington 1974).

2 Blackett, N M. and Adams, K. Cell proliferation and the action of cytotoxic agents on haemopoietic tissue. Br J Haemat. *23* 751 758 (1972).

3 Constable, T B. and Blackett, N M. Comparison of effect of four cytotoxic agents on granulocytic and erythroid repopulating ability of rat bone marrow J natn. Cancer Inst. *48*. 941–948 (1972)

4 Goodman, R. Grate, H. E. Hannon, E., and Hellman, S Hematopoietic stem cells: effect of preirradiation, bleeding and erythropoietin on thrombopoietic differentiation. Blood *49* 253–261 (1977)

5 Gregory G J., McCulloch, E. A., and Till, J E. Erythropoietic progenitors capable of colony formation in culture: state of differentiation. J cell. Physiol. *81* 411–420 (1973).

6 Hellman, S., Grate, H. E., and Chaffey J T Effects of radiation on the stem cell compartment to differentiate into granulocytic and erythrocytic progeny Blood *34* 141 156 (1969).

7 Hellman, S. Grate, H. E. Chaffey J T and Carmel, R. Hematopoietic stem cell compartment pattern of differentiation following radiation or cyclophosphamide; in Stohlman, Hemopoietic cellular proliferation, pp. 36–48 (Grune & Stratton, New York 1970)

8 Ichiki, A T and Lange, R. D A method for the concentration of erythropoietin from human urine. Biochem. Med *10* 50–60 (1974).

9 Iscove, N N The role of erythropoietin in regulation of populatio size and cell cycling of early and late erythroid precursors in mouse and bone marrow Cell Tiss. Kinet. *10* 323–334 (1977).

10 Kubanek, B Bock, O Hert, W Bock, E and Harris, E. B Size and prol f ration of stem cell compartment in mice after depression of erythropoiesis in Hemopoietic Stem Cells, Ciba Foundation Symposium, pp. 43–255 (Elsevier Amsterdam 1973).

11 Lajtha, L G Pozzi L V Schofield, R., and Fox, M Kinetic properties of haemopoieti stem cells. Cell Tiss. Kinet. *2* 39–49 (1969).

12 Lange, R. D Simmonds, M. L., and Dibel m, N R. Polycythaemic mice produced by hy

He was treated with orbenin, garamycin, and the fever went down. Therapy with daunomycin and cytosine arabinoside resulted in haematologic remission in February 1977. At that time haemoglobin was 14.2 g/dl, reticulocytes 2.1%, leukocytes 2,800/µl. Differential count: myelocytes 2%, segmented cells 53%, band forms 2%, eosinophils 1%, lymphocytes 45%, monocytes 7%; platelets were 134,000/µl and the bone marrow was hypocellular with 6% blasts and in some, large granules were found. In spite of consolidation therapy with cytosine arabinoside and thioguanine, he relapsed in April 1977. The morphological findings reappeared, including the large cytoplasmatic granules. No remission was obtained with daunomycin and cytosine arabinoside and later with cytoxan, vincristine and prednisone. The patient died in June 1977. Post-mortem examination was not performed.

Case 2

A 59-year-old white female was admitted to the hospital in June 1976, because of fatigue and weakness for the last 2 months prior to admission. On admission she was in no distress. The pulse rate was 78/min, temperature 36.8 °C and blood pressure 110/70. No lymphadenopathy was found and the spleen and liver were not palpable.

Laboratory Findings

ESR 40/55 (Westergren), haemoglobin 13 g/dl, reticulocytes 1%, leukocytes 8,600/µl, differential count: blasts 34%, segmented cells 25%, band forms 3%, eosinophils 3%, lymphocytes 32%, monocytes 1%, and myelocytes 2%. The blasts *found in the peripheral* blood contained large granules in the cytoplasm. Platelet count 140,000/µl, serum urea, sugar, creatinine, bilirubin, alkaline phosphatase, serum transaminases and prothrombin time were within normal values. Muramidase in serum and urine were normal: 3.6 and < 1 µg/ml, respectively. Bone marrow aspiration revealed a hypercellular marrow. The blasts were 90% and their cytoplasm contained large inclusions. Few lymphocytes, megakaryocytes and normoblasts were also seen. Chest X-ray was normal. She did not respond to daunomycin and cytosine arabinoside and no haematological remission was obtained. She expired in November 1977. Post-mortem examination was not performed.

Materials and Methods

Buffy coat and bone marrow cells were obtained by usual techniques. The cells were examined on smears after staining with May-Grünwald-Giemsa. Cytochemical studies comprised PAS for glycogen [8], methyl green pyronin (MGP) for nucleic acids [13], α-naphthyl acetate esterase [19], naphthol AS-D chloroacetate esterase [17] and acid phosphatase [11]. Electron microscopic studies were performed on buffy coat cells in both cases. Cells were fixed in paraformaldehyde-glutaraldehyde [10], post-fixed in 1.0% OsO_4 in S-collidine buffer [2], dehydrated and embedded according to *Luft* [15]. Sections were cut with LKB Ultrotome III, stained with 0.25% uranyl acetate in a 1:1 solution of H_2O methanol and post-stained with lead citrate [18].

Results

Light Microscopy

Morphology. Unusual cytoplasmatic inclusions were observed in blasts of peripheral blood, as well as of bone marrow. These inclusions differed in size and shape and were solitary or grouped in clusters (fig. 1A). The inclusions often consisted of huge round, homogeneous granules, sometimes irregular in shape. In some cells the inclusions were found in large vacuoles (fig. 1B). In May-Grünwald-Giemsa stain these granules appeared azurophil. A few mitotic cells also contained inclusions (fig. 1C). In peripheral blood of case 2 similar granules were encountered in a few lymphocytes.

Cytochemistry. The granules were PAS-positive and negative in MGP staining. Activity of peroxidase was occasionally observed. No activity of esterase was found neither on α-naphthyl acetate as substrate nor when naphthol AS-D chloroacetate was

Acta haemat. 61 264–271 (1979)

Pseudo-Chediak-Higashi Anomaly in Acute Myeloid Leukaemia

An Electron Microscopical Study

P. Efrati, E. Nir, H. Kaplan and A. Dvilanski

Laboratory for Blood Morphology and Cytology Kaplan Hospital, Rehovot and Soroka Medical Center Ben Gurion University, Beer-Sheva

Key Words. Pseudo-Chediak · Inclusion bodies · Lysosomes · AML

Abstract. 2 cases of acute myeloid leukaemia with inclusion bodies are presented. The inclusions were found mainly in the blast cells but could also be encountered in lymphocytes and plasma cells. Cytochemical and ultrastructural studies showed a great resemblance of these inclusions to the ones found in Chediak-Higashi anomaly, i.e. high acid phosphatase activity, varying in size of inclusions from clusters of small granules to huge inclusion sometimes found in vacuoles, featuring fusion of lysosomes.

Pseudo-Chediak-Higashi anomaly in acute myeloid leukaemia is quite rare [9, 21]. Various inclusion bodies in blasts of acute leukaemias were occasionally described [1, 12, 14, 16]. In the present study 2 cases of actue myeloid leukaemia with inclusion imitating Chediak-Higashi inclusions are described. Morphological, cytochemical and ultrastructural investigations were carried out.

Presentation of Cases

Case 1

A 26-year-old white male was admitted to the hospital in December 1976, because of fever and cervical lymphadenopathy during the last week prior to admission. Past history was noncontributory. On admission he was in good physical condition, pale and in no distress. The pulse rate was 104/min, temperature 40 °C and blood pressure 150/80. Bilateral cervical lymphadenopathy was found and the tip of the spleen was palpable.

Laboratory findings

ESR 91/13 (Westergren), haemoglobin 10.9 g/dl, reticulocytes 2.6%, leukocytes 3,000/µl, differential count – blasts 64%, segmented cells 5%, band forms 1%, myelocytes 4%, promyelocytes 1%, lymphocytes 22%, monocytes 3%. Some blasts in the peripheral blood contained Auer bodies and unusual, large granules. Platelet count 42,000/µl, serum urea, creatinine, sugar, bilirubin, alkaline phosphatase and serum transaminases were within normal limits. Chest X-ray was consistent with pneumonia in the right middle lobe. Marrow aspiration revealed many promyelocytes and blasts, containing the above-mentioned large granules. Marrow karyotype was 46XY without Ph chromosome. Serum and urine muramidase was less than 1 µg/ml (mean normal values for serum and urine muramidase 10.27 and 1.02 µg/ml, respectively).



Fig. 3. Electron microscopy myeloblasts with inclusion bodies × 12,000. A Cell with cytoplasmic electron-dense inclusions (dg). B Clear areas inside electron-dense inclusion ( ). Mitochondria (m) are of different density

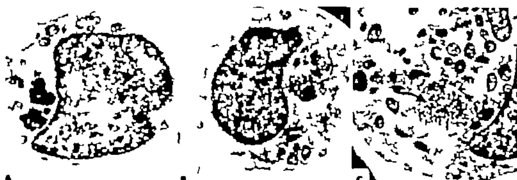


Fig. 4. Electron microscopy myeloblasts with inclusion bodies. A Inhomogeneous irregularly shaped (ir) inclusions 5,000. B clusters of irregular granules (cl) 5,000. C Small dense granules clustered in large vacuole (arrow) mitochondria (m) × 20,000

used. Strong acid phosphatase activity was demonstrated in the inclusions.

Electron Microscopy

In the most immature stage, the blasts had a round or irregular nucleus composed mainly of euchromatin. Heterochromatin – in small amounts – was seen attached to the inner site of nuclear membrane. One or two nucleoli were occasionally encountered. In a later stage of maturity mitochondria appeared in addition (fig. 2A, B). In some



Fig. 1 May-Grünwald-Giemsa staining – acute myeloid leukaemia blasts with giant inclusion bodies. × 800 A Inclusion bodies – solitary or grouped in clusters. B Inclusion bodies in vacuoles C Cell in mitosis with inclusion bodies.



Fig. 2. Electron microscopy – myeloblasts. × 12,000. A Primitive cell Nucleus composed mainly of euchromatin. A small nucleolus (n). B Myeloblast with mitochondria (m) and short profiles of endoplasmic reticulum (er)



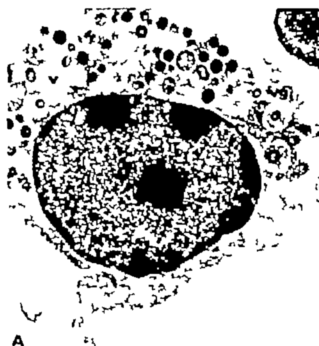
Fig. 7. Electron microscopy: myeloblast with inclusions made of cytoplasma-like material (cg). Mitochondria (m). × 12,000.

cells, cytoplasmatic inclusions made their appearance. They were of various size and shape: huge, round homogeneous electron-dense particles (fig. 3A) sometimes with clear areas inside (fig. 3B). The granules were often irregularly shaped, unhomogeneous (fig. 4A) and accumulated in clusters (fig. 4B). Rarely were they very small, presumably before merging into large granules (fig. 4C). Occasionally the granules were observed within vacuoles (fig. 5A, B). These structures were also seen in later stages of maturation as in myelocytes (fig. 5A), or even in mature granulocytes (fig. 5B). Very rarely were similar granules found in non-myeloid cells, like in plasma cells (fig. 6A) and lymphocytes. Granules found in reticulocytes (fig. 6B) could of course be made of ferritin aggregates. An unusual phenomenon was the appearance of inclusions made of cytoplasma-like material in huge vacuoles (fig. 7).

Discussion

Cytoplasmic inclusions were described in lymphoblastic [3, 1–14], promyelocytic [16] and monocytic [7] leukaemias. In cases of acute myeloid leukaemia such features are quite rare [4, 9, 1].

In the 'Chediak-Higashi' anomaly studied in our laboratory, similar inclusions were found [5, 6]. These inclusions were



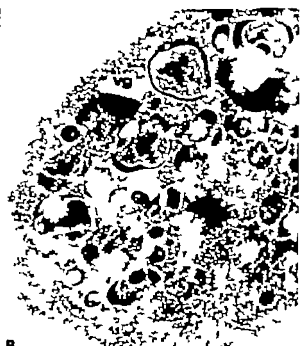


Fig. 5 Electron microscopy – inclusion bodies within vacuoles (vg). A Myelocyte with vacuoles (v) and granules within vacuoles (vg). × 12,000. B Mature granulocyte. × 18,000.





Fig. 6. Electron microscopy – cells of non-*myeloid origin* with inclusion bodies. A Plasma cell with typical endoplasmic reticulum (er) and irregular granule within a vacuole (vg). × 15,000. B Reticulocyte with dense granul (g) × 12,000.

16 Mintz, U Djaldetti, M., Rozenszajn, L. Pinkhas, J and de Vries, A. Giant lysosome-like structures in promyelocytic leukemia. Ultrastructural and cytochemical observations. Biomed. 19 426–430 (1973).
17 Moloney W C, McPherson, K., and Fliegelman, L. Esterase activity in leukocytes demonstrated by the use of naphthol AS-D chloroacetate substrate. J Histochem. Cytochem 8. 200–207 (1960).
18 Reynolds, E. S The use of lead nitrate at high pH as an electron opaque stain in electron microscopy J Cell Biol. 17 208–212 (1963).
19 Rozenszajn, L., Leibowitz, M., Shoham, D and Epstein, J The esterase activity in megaloblasts, leukemia and normal haematopoietic cells. B J Haemat. 14 605–610 (1968).
20 Tanaka, Y Bell, W and Brindley D Pseudoviral inclusion bodies in acute leukemia. A report of two cases. J natn. Cancer Inst. 38. 629–638 (1967).
21 Van-Slyck, E. and Rebuck, J Pseudo-Chediak Higashi anomaly in acute leukemia. Am J clin. Path. 62. 673–678 (1974).

Received. October 20, 1978
Accepted: November 15, 1978

P Efrati, Laboratory for Blood Morphology and Cytology Kaplan Hospital, Rehovot (Israel)

thought to be pathologically changed lysosomes. The high activity of acid phosphatase in the inclusions of Chediak anomaly as well as in the present cases strongly support this assumption This is why we also used the term pseudo-Chediak anomaly in our cases

As pointed out the inclusions were of variable shape and size and sometimes appeared within vacuoles On cytochemical investigations the inclusions were positive for PAS stain and negative for MGP and esterases Staining for acid phosphatase revealed high activity confirming the lysosomal nature of the inclusion [3 20] The finding of clusters of small granules among huge ones is suggesting that the inclusions are formed by fusion of lysosomes

As described above, inclusions were found also in some lymphocytes in the present cases, as well as in the real Chediak anomaly In addition inclusions were observed in some plasma cells. The cause of appearance of these inclusions in acute myeloid leukaemia is not known There is not enough information as to the clinical course in these cases.

Acknowledgement

The authors wish to thank Mr *Y Shimron* for his excellent assistance in photography

References

1 Ackerman, A. G Morphology and cytochemistry *of the granule – vacuole body of leukemic* cells. Blood *12* 443–447 (1957)

2 Bennett, H H and Luft, J H S-collidine as a basis for buffering fixatives. J biophys. biochem. Cytol. *6* 113–114 (1959)

3 Berrebi, A., Oberling, F Mayer S. et Wartz, R., Inclusions intra-cytoplasmiques pseudovi-*rales dans un cas de leucémie aiguë lymphoïde.* Nouv Presse méd. *14* 894 (1974).

4 Brunning, R. D., Parkin, J., Dick, F and Nesbit, M Unusual inclusions occurring in the blasts of four patients with acute leukemia and Down s syndrome. Blood *44* 735–741 (1974)

5 Efrati P and Jonas, W., Chediak's anomaly of leukocytes in malignant lymphoma associated with leukemic manifestations: case report with necropsy Blood *13* 1063–1073 (1958).

6 Efrati P and Danon, D Electron-microscopical study of bone marrow cells in a case of Chediak Higashi Steinbrinck syndrome. Br J Haemat. *15* 173–176 (1968).

7 Freeman, A. and Journey L. J Ultrastructural studies on monocytic leukemia. Br J Haemat. *20* 225–231 (1974).

8 Garton, G., Brandt, L., Franzen, S., and Norden, A. Cytochemical variants of neutrophil leukocyte population in chronic myelocytic leukemia. Scand J Haematol *6* 365–372 (1969).

9 Gorman, A. M. and O'Connell, L. G Pseudo Chediak Higashi anomaly in acute leukemia. Am. J clin. Path. *65* 1030–1031 (1976).

10 Karnovsky M. J. A *formaldehyde-glutaral*dehyde fixative of high osmolarity for use in electron microscopy J Cell Biol *27* 137 138 (1965).

11 Katayama, I. Li, C., and Yam, L. Histochemical study of acid phosphatase isoenzyme in leukemic reticuloendotheliosis. Cancer *29* 157–164 (1972)

12 Komiyama, A. Ogawa, M. Eurenius, K., and Spicer S. S. Unusual cytoplasmic inclusions in blast cells in acute leukemia. Archs Path *100* 590–594 (1976)

13 Kurnick, N B Histological staining with methyl-green pyronin. Stain Technol *27* 233–242 (1952).

14 Lejeune, F., Turpin, F et Lortholary P Inclusions anormales dans les blastes d'une trans*formation aiguë* de leucémie myéloïde chronique Path. Biol., Paris *26* 7 11 (1978)

15 Luft, J H. Improvement in epoxyresin embedding methods. J biophys. biochem. Cytol *9* 409–414 (1961).

16 Mintz, U Djaldetti, M., Rozenszajn, L., Pinkhas, J and de Vries, A. Giant lysosome-like structures in promyelocytic leukemia. Ultrastructural and cytochemical observations. Biomed. *19* 426–430 (1973)
17 Moloney W C., McPherson, K., and Fliegelman, L. Esterase activity in leukocytes demonstrated by the use of naphthol AS-D chloroacetate substrate. J Histochem. Cytochem. *8* 200–207 (1960).
18 Reynolds, E. S The use of lead nitrate at high pH as an electron opaque stain in electron microscopy J Cell Biol. *17* 208–212 (1963).
19 Rozenszajn, L., Leibowitz, M., Shoham, D and Epstein, J The esterase activity in megaloblasts, leukemia and normal haematopoietic cells. Br J Haemat. *14* 605–610 (1968)
20 Tanaka, Y Bell, W and Brandley D Pseudoviral inclusion bodies in acute leukemia. A report of two cases J natn. Cancer Inst. *38*. 629–638 (1967).
21 Van-Slyck, E. and Rebuck, J Pseudo-Chediak-Higashi anomaly in acute leukemia. Am J clin Path. *62*. 673–678 (1974).

Received: October 20, 1978
Accepted November 15, 1978

P Efrati, Laboratory for Blood Morphology and Cytology Kaplan Hospital, Rehovot (Israel)

Acta haemat. *61* 272–277 (1979)

Biosynthetic Studies and γ-Chain Composition in the Greek Type of Hereditary Persistence of Fetal Hemoglobin and in Its Association with β-Thalassemia

C Camaschella M A Ciocca Vasino A Guerrasio G Balegno
E Barberis D Delponte and G Saglio

Istituto di Medicina Interna Cattedra di Clinica Medica II, and Clinica Pediatrica II, Università di Torino, Torino

Key Words. Hereditary persistence of fetal hemoglobin β-Thalassemia Hb F γ-chains Globin synthesis

Abstract. Hematological data biosynthetic studies and γ-chain structure of three heterozygotes for HPFH Greek type and of two heterozygotes for both HPFH and β-thalassemia are reported

In the HPFH heterozygotes, hematological data were normal and globin chain synthesis balanced, while subjects carrying both HPFH and β thalassemia presented a thalassemic picture and the same degree of α/non-α-chain imbalance as the β-thalassemia carrier belonging to the same family The γ-chain composition studies showed only the presence of $^{A}\gamma$-chains in HPFH in the association HPFH/β thalassemia also some $^{G}\gamma$ and ^{T} were found The mechanisms determining the high production of Hb F in the association HPFH/β-thalassemia are discussed

Hereditary persistence of fetal hemoglobin (HPFH) is a condition characterized by fetal hemoglobin synthesis during adult life in subjects who do not show any hemtaological abnormality Two main forms of HPFH have been described. the Negro and the Greek type. In the Negro type the heterozygote shows Hb F ranging between 15 and 30%, while the homozygote presents 100% Hb F [7 14 23 27] In the Greek type only the heterozygote has been so far observed in this form the Hb F is about 15 % of the total hemoglobin [8] In both these conditions the Hb F is homogeneously distributed among the erythrocytes this property was formerly considered to discriminate between HPFH and β-thalassemia on the other hand, some types of HPFH with low percentages of Hb F and heterogeneous distribution of this hemoglobin among the erythrocytes have been described [2, 12 19 20 28]

Since *Schroeder et al* [24] have demonstrated that the γ-chains are codified by two different structural genes, carrying glycine or alanine respectively at position 136 ($^{G}\gamma$ and $^{A}\gamma$) the composition of fetal hemo-

globin in HPFH has been carefully studied. In this way the Negro type has been classified into several subgroups on the basis of γ-chains produced, most commonly both $^G\gamma$ and $^A\gamma$ in different ratios are present, but a form with only $^G\gamma$ chains has been reported [10–13]. In the Greek form all the γ-chains are of the $^A\gamma$ type [11].

Recently *Ricco et al.* [22] have described a new type of γ-chain carrying threonine instead of isoleucine at position 75 (γ^{75Thr}), which is probably the product of a γ-gene ($^T\gamma$), different from $^G\gamma$ and $^A\gamma$ previously reported. Up to now no HPFH Negro type has been studied regarding the 75th residue, while the Greek heterozygote does not seem to have $^T\gamma$-chains [22].

In this paper we report the globin chain synthesis and the Hb F composition in three heterozygotes for HPFH Greek type and in two heterozygotes for both HPFH and β-thalassemia, belonging to the same family.

Materials and Methods

Blood cell counts, hemoglobin electrophoresis and globin chain synthesis were performed in all subjects of the family. The glycine/alanine ratio at position 136 and the $^T\gamma$ percentage were studied in all subjects but I 2, because of technical difficulties in preparing enough amounts of Hb F from a subject with a low percentage of this hemoglobin.

Hematological studies were carried out with standard methods. Red cell indices were determined using a Coulter S cell counter. Electrophoresis of hemoglobin was performed on cellulose acetate pH 8.6 Tris glycine buffer [18] and quantitation of Hb A_2 was obtained by microcolumn chromatographic methods [21]. The percentage of Hb F was dosed by the alkali denaturation technique [4] and by chromatography in columns of DEAE-cellulose [1]. The alkali denaturation method was also used to obtain enough Hb F to determine the glycine/alanine ratio at position 136 and the $^T\gamma$ percentage. The intracellular distribution of Hb F was determined by the acid elution technique [17].

Globin chain synthesis was studied incubating peripheral blood with ^{3}H-leucine for 120 min [16]. Globin was prepared from the entire hemolysate by acid-acetone precipitation. The α-, β- and γ-chains were separated on CM 52 Cellulose chromatography [6] and the total radioactivity of each type of chain was used to calculate the α/non-α-ratio. After tryptic digestion of separated γ-chains, fingerprints were performed at pH 6.4 according to *Sick et al.* [25]. The percentage of the peptide containing γ^{75Thr} (γTp9) was calculated by eluting in methanol the peptides, previously stained with ninhydrin, and reading spectrophotometrically the eluates at 500 nm [22].

The glycine/alanine ratio at position 136 of the γ-chains was determined after cleavage of 10 mg of whole globin F with CNBr by freeze-drying and the dry residue redissolved in a small amount of distilled water. After centrifugation the clear supernatant was again freeze dried and used for the separation *of the γ CB-3 fragment by finger*print at pH 6.4, as previously described [3, 15]. The amino acid analysis has been performed on an automatic aminoacidanalyser after elution of the peptides with 6 *N* HCl and hydrolysis at 110 °C for 24 h.

Results

The propositus (II 1) is a 22-year-old man from Southern Italy who came to our *attention because of subicterus and slightly* enlarged spleen. The hematological findings of this subject revealed a thalassemic picture because of a mild hypochromic microcytic anemia, anisocytosis, poikilocytosis and target cells on peripheral smear. Reticulocyte count was slightly elevated (4.8%), serum iron was normal and total bilirubin was 1.75 mg%. Hemoglobin electrophoresis showed increased Hb A_2 (4.0%), and 40% Hb F; the presence of Hb F was confirmed by the alkali denaturation method.

Table I Hematological findings and results of chain synthesis studies

Cases	Age years	Sex	RBC 10^{12}/l	Hb g/dl	PCV %	MCV fl	MCH pg	MCHC µg/dl	Retics %
I 1	55	M	5 10	15.9	46	90	31	34.5	0.5
I 2	52	F	5.52	11.9	37.5	67 9	21.5	31 7	4 0
II 1	22	M	5.20	12.2	35	67.3	23 4	34.8	4.8
II 2	21	M	5 60	12.2	39	69 6	21 7	31.3	5.3
II 3	18	F	5.20	15.3	45	86	29 4	34	1.3
II-4	27	F	4 40	13 1	40	90	29.5	32.7	1.0
Normal values									< 2.0

[1] The α/β ratio in normal subjects is expressed as specific activity (cpm/OD).

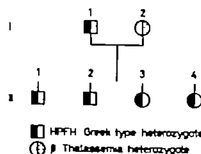


Fig. 1. Pedigree of the family

An hematological examination of the patient s family was then carried out. The pedigree of the family is shown in figure 1 The propositus brother (II 2) showed the same clinical and hematological picture (table I) The father (I 1) and 2 sisters (II 3 and II-4) of the propositus were hematologically normal but presented about 15% Hb F on the electrophoresis.

The mother (I 2) was identified as a β-thalassemia heterozygote on the basis of hemoglobin electrophoresis, red cell indices and globin chain synthesis (α/β ratio=1 96) Globin chain synthesis (expressed as α/non α-ratio of radioactivities) was balanced in

Table II The γ-chains composition in subjects examined

Cases	HbF %	$\gamma 75^{Thr}$ % of the total γ-chains	γ CB₃ glycine	γ CB₃ alanine
I 1	16	0	0.02	3 10
I 2	1.2	–	–	–
II 1	40	8	0.30	2.72
II 2	38	7	0.38	2.60
II 3	15	0	0.05	2.98
II-4	12	0	0 05	3 02

I 1 II 3 and II-4 while in II 1 and II 2 was 1 90 and 1 77 respectively All these data are summarized in table I The Hb F distribution among the erythrocytes in patients I 1 II 3 and II-4 was homogeneous, while in patients II 1 and II 2 two populations of erythrocytes, with low and high percentage of Hb F were present.

Table II reports the types of γ-chains ($^G\gamma$ $^A\gamma$ and $^T\gamma$) found in HPFH and in the association HPFH/β-thalassemia. It must be underlined that in the first case the γ-chains are all of the $^A\gamma$ type, while in HPFH/β-thal

Serum Iron µmol/l	A_2 %	F Betke	Distribut. HbF	$\alpha/(\beta+\gamma)$ cpm
26 23	1.9	16	homogen.	1.05
21 15	4 8	1.2		1.96
29 10	4.0	40	heterog.	1.90
	4.2	38	heterog	1 77
	2 1	12	homogen.	1 11
19 14	2 0	15	homogen.	1.02
M 21 46	2 7	< 1 0		0.98
± 7.51	± 0 8			± 0 10
F 19 14				
± 6.26				

assemia combination also some $^{G}\gamma$ and $^{T}\gamma$ were found.

Discussion

In this paper we report the first Italian carriers of HPFH Greek type up to now this condition has always been described in subjects from Greece and also in our cases we cannot exclude a Greek ancestry since migrations from Greece to Southern Italy were frequent in ancient times.

Subjects heterozygous for HPFH (I 1 II 3 and II–4) have normal hematological data and about 15 / of Hb F entirely of the $^{A}\gamma$ type, homogeneously distributed among the red cells. All these findings are typical of HPFH Greek type and are in keeping with those reported in literature [8, 11 6] As previously reported by *Sofroniadou et al* [6] also in our three cases, globin synthesis gave an 'non-α-ratio within normal values. According to these data, HPFH Greek type at least in the heterozygotes, does not seem a thalassemic condition since the decreased β-chain synthesis is completely balanced by a persistent production of γ-chains. In this way HPFH Greek type differs from HPFH Negro type, in the last condition in fact a slightly unbalanced α/non-α-ratio has been described [5 9 23]

Subjects carrying both HPFH and β-thalassemia (II 1 and II 2) showed the same hematological picture and the same degree of α/non-α-chain imbalance as the β-thalassemia heterozygote belonging to the same family (I 2) This finding is in agreement with the data in the literature [26] On the other hand it must be underlined that in the β-thalassemia trait the production of Hb A attains 12 g* , while in the association reaches only 7 g, the remaining hemoglobin being formed by Hb F In this way the 'non-α-ratio can reach the values observed in the β-thalassemia heterozygote only by remarkably increasing the γ-chain synthesis. These data mean that the β-gene on the HPFH chromosome is not able to compensate the deficiency in β-chain synthesis, as a normal gene does, when a β-thalassemia gene is present in trans. As reported in table II in the HPFH/β-thalassemia combination, the γ-chain analysis showed that, besides $^{A}\gamma$ also γ and low percentages of $^{T}\gamma$ were present. In order to evaluate the amount of γ-chains produced by the chromosome carrying the β-thalassemia gene in the combination HPFH/β-thalassemia, it would be very helpful to examine the 136 Gly/Ala ratio in case I ‑, carrier of β-thalassemia. unfortunately this subject presented a little amount of Hb F so this determination could not be carried out. On the other hand, *Sofroniadou et al* [6] have suggested that in the association, the HPFH chromosome is unable to increase the γ-chain synthesis and that the depression of the γ-genes on the

β-thalassemia chromosome is responsible for the increased amount of Hb F the presence of $^{G}\gamma$ and $^{A}\gamma$-chains in II 1 and II 2 can agree with these hypotheses.

In the association HPFH/β-thalassemia all the red cells contain Hb F but the distribution of this hemoglobin is not homogeneous in fact two populations are clearly distinguishable the first contains low amounts of Hb F and the second contains higher amounts of Hb F Presumably this heterogeneity is related to the different synthesis of γ-chains directed by the β thalassemia gene superimposed to the $^{A}\gamma$ produced by the HPFH chromosome

Up to now the genetic defect underlying the HPFH Greek type is not known two hypotheses can be taken into account, either a β-thalassemia condition completely compensated by an increased γ-chain synthesis or an altered mechanism of transcription of the closely linked β- δ- and γ-genes [26] Anyway a β-chain synthesis reduction exists and becomes more evident in the HPFH/β thalassemia association whereas only a marked γ-chain synthesis in trans to the HPFH allows to attain adequate levels of hemoglobin How these genes can increase their expression is still obscure and further data about the regulation of the non α-gene activity are needed in order to clarify this problem

References

1 Abraham, E. C. Reese, A. Stallings, M and Huisman, T H J Separation of hum n hemoglobins by DEAE-cellulose chromatography using glycine KCN NaCl developers. Hemoglobin *1* 27–44 (1976/77)

2 Altay C Huisman, T H J and Schroeder W A. Another form of hereditary persistence of fetal hemoglobin (the Atlanta type)? Hemoglobin *1* 125–133 (1976/77)

3 Bernini L. F Fiorelli, G Mattoonto, B., Bianchi, G e Chiesa, G Polimorfismo strutturale dell Hb Fetale umana. Atti riunioni Gruppo per lo studio dell eritrocita, pp. 25-33 (Cilab Milano 1971)

4 Betke, K. Marti M R and Schlicht, I Estimation of small percentages of fetal hemoglobin Nature, Lond. *184* 1877–1878 (1959).

5 Charache, S Clegg, J B and Weatherall, D. J The Negro variety of hereditary persistence of fetal hemoglobin is a mild form of thalassemia. Br J Haemat. *34* 527–534 (1976).

6 Clegg, J B Naughton, M A., and Weatherall, D J Abnormal human hemoglobin. Separation and characterization of the α and β chains by chromatography and the determination of two new β variants Hb Chesapeake and Hb J (Bangkok) J molec. Biol *19* 91–108 (1966).

7 Conley C. L. Weatherall D J Richardson, S. N Shepard, M K., and Charache S. Hereditary persistence of fetal hemoglobin. a study of 79 affected persons in 15 Negro families in Baltimore. Blood *21* 261–281 (1963).

8 Fessas, P and Stamatoyannopoulos, G Hereditary persistence of fetal hemoglobin in Greece A study and comparison. Blood *24* 223–240 (1964).

9 Friedman, S. Schwartz, E. Ahern, E., and Ahern, V Variations in globin chain synthesis in hereditary persistence of fetal hemoglobin. Br J Haemat *32* 357–364 (1976).

10 Huisman T H. J Schroeder W A. Dozy A. M Shelton, J R Shelton, J B Boyd, E. M and Apell G Evidence for multiple structural genes for the γ chains of human fetal hemoglobin in hereditary persistence of fetal hemoglobin. Ann N Y Acad. Sci *165* 320–331 (1969)

11 Huisman, T H J Schroeder W A. Stamatoyannopoulos, G Bouver N Shelton, J R., Shelton, J B and Apell, G Nature of fetal hemoglobin in the Greek type of hereditary persistence of fetal hemoglobin with and without concurrent β thala semia. J clin. Invest *49* 1035–1040 (1970a)

12 Huisman, T H J Schroeder W A Adams, H R. Shelton, J R Shelton, J B and Apell,

J. A possible subclass of hereditary persistence of fetal hemoglobin. Blood 56: 1-9 (1970b).

13 Huisman, T. H. J.; Schroeder, W. A.; Efremov, G. D.; Duma, H.; Mladenovski, B.; Hyman, C. B.; Rachmilewitz, E. A.; Bouver, N.; Miller, A.; Brodie, A.; Shelton, J. R.; Shelton, J. B., and Apell, G.: The present status of the heterogeneity of fetal hemoglobin in β thalassemia; an attempt to unify some observations in thalassemia and related conditions. Ann. N. Y. Acad. Sci. 232: 107-124 (1974).

14 Kamuzora, H.; Ringelmann, B.; Konotey-Ahulu, F. I. D.; Lehmann, H., and Lorkin, P. A.: The γ chain in Ghanaian adult, homozygous for hereditary persistence of fetal haemoglobin. Acta haemat. 1: 179-184 (1974).

15 Kamuzora, H.; Ringelmann, B.; Konotey-Ahulu, F. I. D.; Lehmann, H., and Lorkin, P. A.: Further investigations of the chain in Ghanaian adult, homozygous for hereditary persistence of fetal haemoglobin. Isolation of γ CB-3 peptides and ratio determination in human Hb F. Acta haemat. 53: 315-320 (1975).

16 Kan, Y. W.; Schwartz, E., and Nathan, D. G.: Globin synthesis in the thalassemia syndromes. J. clin. Invest. 47: 2515-2522 (1968).

17 Kleihauer, E.; Brown, H. and Betke, K.: Demonstration von fetalem Hämoglobin in den Erythrocyten eines Blutausstriches. Klin. Wschr. 35: 635-636 (1957).

18 Marengo-Rowe, A. J.: Rapid electrophoresis and quantitation of haemoglobins on cellulose acetate. J. clin. Path. 18: 790-795 (1965).

19 Martin, H. R.: Normale und anormale menschliche Hämoglobine, pp. 81-93 (Springer, Berlin 1963).

20 Martinez, G. and Colombo, B.: A new type of hereditary persistence of fetal hemoglobin. I. diffusible factor regulating β chain synthesis. Nature, Lond. 252: 735-736 (1974).

21 Nastev, D.; Efremov, G. D., and Petkov, G.: A test tube method for quantitation of hemoglobin A₂ using DE cellulose. Hemoglobin 1: 445-459 (1977).

22 Russo, G.; Mazza, U.; Turi, R. M.; Pich, P. G.; Camaschella, C.; Saglio, G. and Bernini, L. F.: Significance of new type of human fetal hemoglobin carrying replacement isoleucine → threonine at position 75 (E19) of the γ chain. Hum. Genet. 32: 305-313 (1976).

23 Ringelmann, B.; Acquaye, C. T. A.; Oldham, J. H.; Konotey-Ahulu, F. D. I.; Yawson, G.; Sukumaran, P. K.; Schroeder, W. A., and Huisman, T. H. J.: Homozygotes for the hereditary persistence of fetal hemoglobin: the ratio of $^{G}\gamma$ to $^{A}\gamma$ chains and biosynthetic studies. Biochem. Genet. 15: 1083-1096 (1977).

24 Schroeder, W. A.; Huisman, T. H. J.; Shelton, R. J.; Shelton, J. B.; Kleihauer, E. F.; Dozy, A. M., and Robberson, B.: Evidence for multiple structural genes for the γ chain of human fetal hemoglobin. Proc. nat. Acad. Sci. USA 60: 537-544 (1968).

25 Sick, K.; Beale, D.; Irvine, D.; Lehmann, H.; Goodhall, P. T., and MacDougall, S.: Haemoglobin G Copenhagen and haemoglobin J Cambridge. Two new β chain variants of haemoglobin A. Biochim. biophys. Acta 140: 231-4 (1967).

26 Sofroniadou, K.; Wood, W.; Nute, P. E., and Stamatoyannopoulos, G.: Globin chain synthesis in the Greek type of hereditary persistence of fetal hemoglobin. Br. J. Haemat. 29: 137-148 (1975).

27 Sukumaran, P. K.; Huisman, T. H. J.; Schroeder, W. A.; McCurdy, P. R.; Freehafer, J. T.; Bouver, N.; Shelton, J. R.; Shelton, J. B. and Apell, G.: A homozygote for the Hb $^{G}\gamma$ type of fetal hemoglobin in India: study of two Indian and four Negro families. Br. J. Haemat. 23: 403-417 (1972).

28 Weatherall, D. J.; Cartner, R.; Clegg, J. B.; Wood, W. G.; MacRae, I. A., and Mackenzie, A.: A form of hereditary persistence of fetal hemoglobin characterized by uneven cellular distribution of haemoglobin F and the production of haemoglobins A and A₂ in homozygotes. Br. J. Haemat. 29: 205-220 (1975).

Received: July 1, 1978
Accepted: August 19, 1978

C. Camaschella, Istituto di Medicina Interna dell'Università di Torino,
Cattedra di Clinica Medica II
Via Genova 3, I 10126 Torino (Italy)

β-thalassemia chromosome is responsible for the increased amount of Hb F the presence of $^{G}\gamma$ and $^{T}\gamma$-chains in II 1 and II 2 can agree with these hypotheses

In the association HPFH/β-thalassemia all the red cells contain Hb F but the distribution of this hemoglobin is not homogeneous in fact two populations are clearly distinguishable the first contains low amounts of Hb F and the second contains higher amounts of Hb F Presumably this heterogeneity is related to the different synthesis of γ-chains directed by the β-thalassemia gene superimposed to the $^{A}\gamma$ produced by the HPFH chromosome

Up to now the genetic defect underlying the HPFH Greek type is not known two hypotheses can be taken into account, either a β-thalassemia condition completely compensated by an increased γ-chain synthesis, or an altered mechanism of transcription of the closely linked β- δ- and γ-genes [26] Anyway a β-chain synthesis reduction exists and becomes more evident in the HPFH/β thalassemia association whereas only a marked γ-chain synthesis in trans to the HPFH allows to attain adequate levels of hemoglobin. How these genes can increase their expression is still obscure and further data about the regulation of the non α-gene activity are needed in order to clarify this problem.

References

1 Abraham, E. C. Reese, A. Stallings, M and Huisman, T H. J. Separation of human hemoglobins by DEAE-cellulose chromatography using glycine KCN N Cl developers. Hemoglobin *1* 27–44 (1976/77).

2 Altay C., Huisman, T H J and Schroeder W A. Another form of hereditary persistence of fetal hemoglobin (the Atlanta type)? Hemoglobin *1* 125–133 (1976/77)

3 Bernini, L. F., Fiorelli, G Mannucci, P Bianchi, G e Chiesa, G Polimorfismo strutturale dell'Hb Fetale umana. Atti riunion Gruppo per lo studio dell'eritrocita, pp. 25–33 (Cilab Milano 1971)

4 Betke, K. Marti, M. R., and Schlicht, L. Estimation of small percentages of fetal hemoglobin. Nature, Lond. *184* 1877–1878 (1959).

5 Charache, S., Clegg, J B and Weatherall, D. J The Negro variety of hereditary persistence of fetal hemoglobin is a mild form of thalassemia. Br J Haemat. *34* 527–534 (1976).

6 Clegg, J B Naughton, M. A., and Weatherall, D J Abnormal human hemoglobin. Separation and characterization of the α and β chains by chromatography and the determination of two new β variants Hb Cheasepeake and Hb J (Bangkok) J molec. Biol. *19* 91–108 (1966).

7 Conley C. L. Weatherall, D J Richardson, S. N., Shepard, M. K., and Charache, S. Hereditary persistence of fetal hemoglobin. a study of 79 affected persons in 15 Negro families in Baltimore Blood *21* 261–281 (1963).

8 Fessas, P and Stamatoyannopoulos, G Hereditary persistence of fetal hemoglobin in Greece. A study and comparison. Blood *24* 223–240 (1964).

9 Friedman, S., Schwartz, E., Ahern, E., and Ahern, V Variations in globin chain synthesis in hereditary persistence of fetal hemoglobin. Br J Haemat. *32* 357–364 (1976).

10 Huisman, T H J Schroeder W A., Dozy A. M Shelton, J R. Shelton, J B Boyd, E. M., and Apell, G Evidence for multiple structural genes for the γ chains of human fetal hemoglobin in hereditary persistence of fetal hemoglobin. Ann. N Y Acad. Sci. *165* 320–331 (1969)

11 Huisman, T H. J Schroeder W A. Stamatoyannopoulos, G Bouver N Shelton, J R., Shelton, J B., and Apell, G Nature of fetal hemoglobin in th Greek type of hereditary persistence of fetal hemoglobin with and without concurrent β thalassemia. J clin. Invest. *49* 1035–1040 (1970a).

1 Huisman, T H. J Schroeder W A. Adams, H. R. Shelton, J R. Shelton, J B and Apell,

J A possible subclass of hereditary persistence of fetal hemoglobin. Blood 36, 1-9 (1970b)

13 Huisman, T H. J Schroeder W A., Efremov G D Duma, H., Mladenovski, B., Hyman, C. B., Rachmilevitz, E A., Bouver N., Miller A., Brodie, A., Shelton, J R., Shelton, J B., and Apell, G The present status of the heterogeneity f fetal hemoglobin in β thalassemia an attempt to unify some observations in thalassemia and related conditions. Ann. N. Y Acad Sci. 232 107-124 (1974).

14 Kamuzora, H., Ringelmann, B Konotey-Ahulu, F I D Lehmann, H., and Lorkin, P A. The γ chain in Ghanaian adult, homozygous for hereditary persistence of fetal haemoglobin. Acta haemat 51 179-184 (1974).

15 Kamuzora, H Ringelmann, B Konotey-Ahulu, F I. D Lehmann, H and Lorkin, P A Further investigations of the γ chain in Ghanaian adult, homozygous for hereditary persistence of fetal hemoglobin. Isolation of γ CB-3 peptides and $^{G}\gamma$ $^{A}\gamma$ ratio determination in human Hb F Acta haemat. 53 315-320 (1975).

16 Kan, Y W Schwartz, E and Nathan, D G Globin synthesis in the thalassemia syndromes J clin. Invest. 47 2515-2522 (1968).

17 Kleihauer, E., Brown, H und Betke, K. Demonstration von fetalem Hämoglobin in den Erythrocyten eines Blutabstrichs. Klin. Wschr 35 635-636 (1957).

18 Marengo-Rowe, A. J Rapid electrophoresis and quantitation of haemoglobins on cellulose acetate J clin Path. 18. 790-795 (1965).

19 Martin, H R. Normale und anormale menschliche Hämoglobine, pp 81-93 (Springer Berlin 1963).

20 Martinez, G and Colombo, B A new type of hereditary persistence of fetal hemoglobin: Is diffusible factor regulating β chain synthesis? Nature, Lond 252 735-736 (1974)

21 Nastev D., Efremov G D and Petkov G A test tube method for quantitation of hemoglobin A_2 using DE 52 cellulose Hemoglobin 1 445-459 (1977).

22 Ricco, G Mazza, U Turi, R M Pich, P G Camaschella, C Saglio, G and Bernini, L. F Significance of new type of human fetal hemoglobin carrying replacement isoleucine → threonine at position 75 (E19) of the γ chain. Hum. Genet. 32. 305-313 (1976).

23 Ringelmann, B Acquaye, C. T A Oldham, J H., Konotey-Ahulu, F D. L, Y ason, G.; Sukumaran, P K., Schroeder W A., and Huisman, T H. J Homozygotes for the hereditary persistence of fetal hemoglobin. the ratio of $^{G}\gamma$ to $^{A}\gamma$ chains and biosynthetic studies. Biochem. Genet. 15 1083-1096 (1977)

24 Schroeder W A., Huisman, T H. J Shelton, R. J Shelton, J B., Kleihauer E. F Dozy A. M and Robberson, B Evidence for multiple structural genes for the γ chain of human fetal hemoglobin. Proc. nat. Acad. Sci. USA 60 537-544 (1968).

25 Sick, K., Beale, D Irvine, D., Lehmann, H., Goodhall, P T and MacDougall, S. Haemoglobin G Kopenhagen and haemoglobin J Cambridge Two new β chain variants of hemoglobin A. Biochim. biophys. Acta 140-231 242 (1967).

26 Sofroniadou, K., Wood, W Nute, P E., and Stamatoyannopoulos, G Globin chain synthesis in the Greek type of hereditary persistence of fetal hemoglobin. Br J Haemat. 29-137 148 (1975).

27 Sukumaran, P K Huisman, T H. J Schroeder W A., McCurdy P R Freehafer J T Bouver N Shelton, J R., Shelton, J B and Apell, G. A homozygote for the Hb $^{G}\gamma$ type of fetal hemoglobin in India. study of tw Indian and four Negro families. Br J Haemat. 23 403-417 (1972)

28 Weatherall, D J Cartner R., Clegg, J B., Wood, W G MacRae, I. A., and MacKenzie, A A form of hereditary persistence of fetal hemoglobin characterized by uneven cellular distribution of haemoglobin F and the production of haemoglobins A and A_2 in homozygotes Br J Haemat 29 205-220 (1975)

Received: July 21 1978
Accepted: August 18, 1978

C. Camaschella, Istituto di Medicina Interna dell'Università di Torino,
Cattedra di Clinica Medica II,
Via Genova 3, I-10126 Torino (Italy)

β-thalassemia chromosome is responsible for the increased amount of Hb F the presence of $^G\gamma$ and $^T\gamma$-chains in II 1 and II 2 can agree with these hypotheses.

In the association HPFH/β thalassemia all the red cells contain Hb F but the distribution of this hemoglobin is not homogeneous in fact two populations are clearly distinguishable the first contains low amounts of Hb F and the second contains higher amounts of Hb F Presumably this heterogeneity is related to the different synthesis of γ-chains directed by the β thalassemia gene superimposed to the $^A\gamma$ produced by the HPFH chromosome

Up to now the genetic defect underlying the HPFH Greek type is not known two hypotheses can be taken into account, either a β-thalassemia condition, completely compensated by an increased γ-chain synthesis, or an altered mechanism of transcription of the closely linked β- δ- and γ-genes [26] Anyway a β-chain synthesis reduction exists and becomes more evident in the HPFH/β-thalassemia association whereas only a marked γ-chain synthesis in trans to the HPFH allows to attain adequate levels of hemoglobin How these genes can increase their expression is still obscure and further data about the regulation of the non α-gene activity are needed in order to clarify this problem.

References

1 Abraham, E. C Reese, A. Stallings, M., and Huisman, T H. J Separation of human hemoglobins by DEAE-cellulose chromatography using glycine-KCN NaCl developers. Hemoglobin *1* 27–44 (1976/77)

2 Altay C. Huisman, T H. J and Schroeder W A.. Another form of hereditary persistence of fetal hemoglobin (the Atlanta type) Hemoglobin *1* 125–133 (1976/77)

3 Bernini, L F Fiorelli, G Marinoni, B Bianchi G e Chiesa, G Polimorfismo strutturale dell'Hb Fetale umana. Atti numero Gruppo per lo studio dell'eritrocita, pp. 25-33 (Cilab, Milano 1971).

4 Betke, K., Marti, M R., and Schlicht, L. Estimation of small percentages of fetal hemoglobin. Nature, Lond. *184* 1877–1878 (1959).

5 Charache, S., Clegg, J B and Weatherall, D J The Negro variety of hereditary persistence of fetal hemoglobin is a mild form of thalassemia. Br J Haemat. *34* 527–534 (1976).

6 Clegg, J B Naughton M A., and Weatherall, D J Abnormal human hemoglobin. Separation and characterization of the α and β chains by chromatography and the determination of two new β variants Hb Cheasepeake and Hb J (Bangkok) J molec. Biol *19* 91–108 (1966).

7 Conley C L., Weatherall, D J Richardson, S. N Shepard, M. K., and Charache, S Hereditary persistence of fetal hemoglobin: a study of 79 affected persons in 15 Negro families in Baltimore. Blood *21* 261–281 (1963).

8 Fessas, P and Stamatoyannopoulos, G Hereditary persistence of fetal hemoglobin in Greece. A study and comparison. Blood *24* 223–240 (1964).

9 Friedman, S Schwartz, E. Ahern, E., and Ahern, V Variations in globin chain synthesis in hereditary persistence of fetal hemoglobin. Br J Haemat. *32* 357–364 (1976).

10 Huisman, T H J Schroeder W A Dozy A. M Shelton, J R. Shelton, J B Boyd, E. M and Apell, G Evidence for multiple structural genes for the γ chains of human fetal hemoglobin in hereditary persistence of fetal hemoglobin. Ann N Y Acad. Sci. *165* 320–331 (1969)

11 Huisman, T H J Schroeder W A Stamatoyannopoulos, G Bouver N Shelton, J R., Shelton, J B., and Apell, G Nature of fetal hemoglobin in the Greek type of hereditary persistence of fetal hemoglobin with and without concurrent β thalassemia. J Clin Invest. *49* 1035–1040 (1970a).

12 Huisman, T H J Schroeder W A. Adams, H R., Shelton, J R., Shelton, J B and Apell,

tomy and was followed closely for many months before and after the operation the poor tolerance of pregnancy and infections has been noted in others.

Materials and Methods

Routine haematological indices were measured by Coulter model-S counter Haemoglobin electrophoresis was carried out on cellulose acetate membranes using TEB buffer pH 8.4 Staining was done with ponceau-S or o-dianisidine to differentiate free α-chains from the carbonic anhydrase enzymes. Hb A_2 and free α-chains were quantitated by elution from unstained cellulose acetate strips and spectrophotometry Hb F was quantitated by the alkali denaturation method of *Betke et al.* [1] except for values over 5% when the technique of *Singer et al* [9] was used Haemoglobin stability was assessed by the heat-denaturation test of *Dacie et al* [3] and the isopropanol method of *Carrell and Kay* [2].

To identify the abnormality in the unstable haemoglobin the haemolysate was treated as in the diagnostic heat stability test but the exposure to 50 C was carried out not for 1 h but for 20 min only to avoid any possible co-precipitation of Hb A The precipitated haemoglobin was then analysed following the techniques summarised by *Sick et al.* [8]. Globin was separated from the haem and digested with trypsin. The tryptic peptides were then separated by high-voltage electrophoresis in one direction and at right angles to this direction by chromatography The resulting two-dimensional peptide chromatogram ("fingerprint") was stained with ninhydrin. The area where β Tp III (residues 18–30) is usually found showed no peptide spot. There was, however, below this area peptide which is not present in fingerprints of Hb A which had the same electrophoretic mobility as β Tp III. On amino acid analysis it was found to contain all the amino acids found in β Tp III, with the exception that one leucine residue was missing and that there was one additional proline residue. As the only leucine residue in β Tp III is β28 the results suggested that the mutation was β28 Leu→Pro, i.e. that the abnormal variant was Hb Genova.

Results

The family pedigree is shown in figure 1 and pertinent haematological data in table I. Most of the affected members suffer from a moderately severe anaemia with brisk reticulocytosis exceptionally the oldest affected member (II 2) maintains a reasonably good haemoglobin level and denies any disability The slight Hb F elevations are in keeping with other reports, as are the increases in Hb A_2. The consistent finding of free α-chains on haemoglobin electrophoresis, along with reticulocytosis, allowed easy separation of affected individuals during the in-

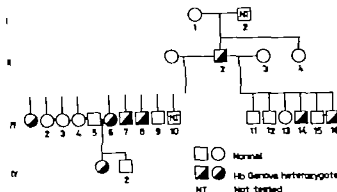


Fig. 1. Family pedigree

Acta haemat. 61 278–282 (1979)

The Unstable Hb Genova (β28Leu→Pro) in an East African Family

Family Study and the Effect of Splenectomy

A. Kendall, S. Young, N. Oune, B. Wiltshire and H. Lehmann

Department of Medicine, University of Nairobi; Department of Pathology, Kenyatta National Hospital, Nairobi, and Department of Biochemistry, University of Cambridge, Cambridge

Key Words. Hb Genova · Haemoglobinopathies · Unstable haemoglobins · Splenectomy · Kenya

Abstract. A fifth family with the unstable Hb Genova is reported from Kenya, East Africa. This almost certainly represents an independent mutation. The beneficial effect of splenectomy has been carefully assessed in one member of the family.

By January 1977 some 56 unstable haemoglobins had been described. However, since the criteria of instability are not well defined, this number remains an approximation. Of the unstable haemoglobins described, 9 owe their instability to the substitution of proline for either leucine or alanine in either the α- or β-globin chain. One of these 9, Hb Genova (β28 Leu→Pro) has been reported in 4 families, all of European background [5–7, 10, 11].

In these 4 affected families the unstable haemoglobin has been roughly quantitated at 10–25% of the total haemoglobin in the circulating erythrocytes. Hb A_2 has been elevated in a few, but not all cases. Hb F has been normal or, more usually, slightly increased; reticulocytosis has been uniformly present, free α-chains have usually been detectable by haemoglobin electrophoresis, but Hb Genova itself migrates with Hb A. In the affected individuals, splenomegaly has generally been found. Three underwent splenectomy, one because of traumatic rupture of the organ at an early age, and two long before the nature of the haemolytic disease was known. While it is stated that one patient showed no improvement after splenectomy, another is said to have undergone the operation 'with satisfactory results'. It is clear that the value of splenectomy in these cases could not be properly assessed.

In the present report an indigenous Kenyan family of the Kikuyu tribe is described in which 8 members have been found to possess Hb Genova. The family was initially discovered by one of us (S.Y.) some 10 years ago and the identification of the haemoglobin variant was made in the MRC Abnormal Haemoglobin Unit, Cambridge. Since that time family studies have been extended; one member has undergone splenec-

tomy and was followed closely for many months before and after the operation the poor tolerance of pregnancy and infections has been noted in others.

Materials and Methods

Routine haematological indices were measured by Coulter model-S counter. Haemoglobin electrophoresis was carried out on cellulose acetate membranes using TEB buffer pH 8.4. Staining was done with ponceau-S or o-dianisidine to differentiate free α-chains from the carbonic anhydrase enzymes. Hb A_2 and free α-chains were quantitated by elution from unstained cellulose acetate strips and spectrophotometry. Hb F was quantitated by the alkali denaturation method of *Betke et al.* [1] except for values over 5% when the technique of *Singer et al.* [9] was used. Haemoglobin stability was assessed by the heat-denaturation test of *Dacie et al.* [3] and the isopropanol method of *Carrell and Kay* [2].

To identify the abnormality in the unstable haemoglobin the haemolysate was treated as in the diagnostic heat stability test but the exposure to 50 C was carried out not for 1 h but for 20 min only to avoid any possible co-precipitation of Hb A. The precipitated haemoglobin was then analysed following the techniques summarised by *Sick et al.* [8]. Globin was separated from the haem and digested with trypsin. The tryptic peptides were then separated by high-voltage electrophoresis in one direction and at right angles to this direction by chromatography. The resulting two-dimensional peptide chromatogram ('finger print') was stained with ninhydrin. The area where β^Tp III (residues 18–30) is usually found showed no peptide spot. There was, however, below this area a peptide which is not present in fingerprints of Hb A which had the same electrophoretic mobility as β^Tp III. On amino acid analysis it was found to contain all the amino acids found in β^Tp III, with the exception that one leucine residue was missing and that there was one additional proline residue. As the only leucine residue in β Tp III is β28 the results suggested that the mutation was β28 Leu→Pro, i.e. that the abnormal variant was Hb Genova.

Results

The family pedigree is shown in figure 1 and pertinent haematological data in table I. Most of the affected members suffer from a moderately severe anaemia with brisk reticulocytosis exceptionally the oldest affected member (II 2) maintains a reasonably good haemoglobin level and denies any disability. The slight Hb F elevations are in keeping with other reports, as are the increases in Hb A_2. The consistent finding of free α-chains on haemoglobin electrophoresis, along with reticulocytosis, allowed easy separation of affected individuals during the in-

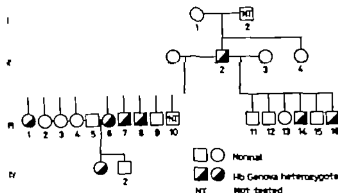


Fig. 1. Family pedigree.

Acta haemat. 61 278–282 (1979)

The Unstable Hb Genova (β28Leu→Pro) in an East African Family

Family Study and the Effect of Splenectomy

A Kendall S Young N Oune B Wiltshire and H Lehmann

Department of Medicine, University of Nairobi Department of Pathology
Kenyatta National Hospital, Nairobi, and Department of Biochemistry University of Cambridge, Cambridge

Key Words. Hb Genova Haemoglobinopathies Unstable haemoglobins Splenectomy Kenya

Abstract A fifth family with the unstable Hb Genova is reported from Kenya, East Africa. This almost certainly represents an independent mutation. The beneficial effect of splenectomy has been carefully assessed in one member of the family

By January 1977 some 56 unstable haemoglobins had been described. However since the criteria of instability are not well defined, this number remains an approximation Of the unstable haemoglobins described, 9 owe their instability to the substitution of proline for either leucine or alanine in either the α- or β-globin chain One of these 9 Hb Genova (β28 Leu→Pro) has been reported in 4 families, all of European background [5–7 10 11]

In these 4 affected families the unstable haemoglobin has been roughly quantitated at 10–25% of the total haemoglobin in the circulating erythrocytes Hb A_2 has been elevated in a few but not all, cases Hb F has been normal or more usually slightly increased reticulocytosis has been uniformly present free α-chains have usually been detectable by haemoglobin electrophoresis, but Hb Genova itself migrates with Hb A. In the affected individuals, splenomegaly has generally been found Three underwent splenectomy one because of traumatic rupture of the organ at an early age, and two long before the nature of the haemolytic disease was known. While it is stated that one *patient showed no improvement* after splenectomy another is said to have undergone the operation 'with satisfactory results It is clear that the value of splenectomy in these cases could not be properly assessed.

In the present report an indigenous Kenyan family of the Kikuyu tribe is described in which 8 members have been found to possess Hb Genova. The family was initially discovered by one of us *(S Y)* some 10 years ago and the identification of the haemoglobin variant was made in the MRC Abnormal Haemoglobin Unit, Cambridge. Since that time family studies have been extended one member has undergone splenec

aged 12.8 g/dl. *Symptomatically he seems* much improved and is functioning physically at a higher level.

Discussion

In other reports of families with unstable haemoglobins it has been suggested that there may be variable expression of the abnormal gene. Certainly in the present family the 8 affected members show a *fairly* consistent haematological picture with the possible exception of the oldest member (II 2) who appears less affected or has achieved better compensation. Many factors influence an individual's response to an undesirable gene such as that responsible for Hb Genova. These include the compensatory capacity of the bone marrow the functioning of the reticulo-endothelial system, the tolerance of the tissues to hypoxaemia, and *the oxygen affinity of the abnormal haemoglobin.*

Splenectomy has frequently been performed on patients with unstable haemoglobins, with variable results. In contemplating this procedure the possible advantages must be weighed against the hazards of the asplenic state and the extra burden imposed on the liver Our splenectomized patient (III 7) is perhaps the first case with Hb Genova in whom the diagnosis was known pre-operatively and who has been *followed closely both before and after splenectomy* This evaluation permits the conclusion that removal of the spleen, in this case at least, has been highly beneficial.

At least 10 different haemoglobin variants have been found in the indigenous population of Kenya [4 *Kendall* to be published] Most of these have occurred in the *Nilotic Luo* and western *Bantu* Abaluhya populations. To date there have been virtually no variants detected in the central *Bantu Kikuyu tribe, the most numerous population group in the country* Thus the finding of the rare variant Hb Genova in a pure Kikuyu family is of special interest. In the 4 European families with Hb Genova, the origins have been Italian, French and Ukrainian. Based on family studies one individual may have acquired the anomalous gene as a new mutation *[10]* Almost certainly Hb Genova in our Kenyan family represents an independent mutation.

References

1 Betke, K Marti, H. R., and Schlicht, L. Estimation of small percentages of foetal haemoglobin. Nature, Lond. *184* 1877–1878 (1959).

2 Carrell, R W and Kay R.. A simple method for the detection of unstable haemoglobins. Br J Haemat. 23. 615–619 (1972).

3 Dacie, J V *Grimes*, A. J *Meisler* A., Steingold, L., Hepsted, E. H Beaven, G H., and White, J C Hereditary Heinz-body anaemia A report of studies on five patients with mild anaemia. Br J Haemat. *10* 388–402 (1964).

4 Kendall, A. G. and Barr R D Haemoglobinopathies in Kenya Trans. R. Soc trop. Med. Hyg. *67* 770–781 (1973).

5 Labie, D., Bernadou, A., W Jenman, H. et Bilski-Pasquier G Observation familiale d'une hémoglobine Genova β28(B10) Leu→Pro. Etude clinique, hématologique, génétique et biochimique d'une famille française. Nouv Revue fr Hémat. *12* 502–506 (1972).

6 Sansone, G. and Pik, C. Familial haemolytic anaemia with erythrocyte inclusion bodies, bilifuscinuria and abnormal haemoglobin (Haemoglobin Galliera Genova). Br J Haemat. *11* 511 517 (1965).

7 Sansone, G Carrell, R. W and Lehmann, H.: Haemoglobin Genova β28(B10) Leucine→proline. Nature, Lond. *214* 877–879 (1967).

Table I. Haematological data

Subject	Age years	Hb g/dl	Reticulocytes %	MCV fl	Hb F %	Hb A_2 %	Free α-chains (% or +)
Affected subjects							
II 2	~60	11.4	22	95	0.8	4.9	1.3
III 1	34	7.9	20	89	2.4	3.3	+
III-6	28	8.8	23	87	1.8	4.5	1.7
III 7	26	7.1	23	92	1.7	4.6	1.2
III-8	18	9.3	24	85	1.7	5.0	1.0
III 14	4	9.0	17	89	2.0	4.3	+
III 16	10	8.5	17	86	3.8	4.9	+
IV 2	3½	9.2	22	88	6.1	3.8	+
Unaffected subjects							
I 1	~90	12.8	0	82	–	–	–
II-4	~45	13.7	2	98	–	–	–
III 2	31	12.9	3	91	–	–	–
III 3	25	11.9	1	93	–	3.3	–
III-4	21	14.9	2	99	–	–	–
III 9	15	14.9	2	84	0.2	–	–
III 11	2	11.8	2	85	–	–	–
III 12	13	15.2	1	94	–	3.3	–
III 13	6	14.3		94	–	–	–
III 15	12	14.4	0	95	–	–	–
IV 3	5	13.4	4	79	0.3	–	–
Splenectomized subject (III 7)							
Pre-splenectomy		7.1	23				
Post-splenectomy		12.8	13				

itial family screening. Clinically mild splenomegaly a regular finding in the affected individuals, also facilitated their segregation. Heat denaturation and isopropanol stability tests were unequivocally positive in all affected members.

Several individuals were observed through pregnancy and during acute infections. Despite daily folic acid supplementation their haematologic status usually deteriorated, with an aggravation of the haemolytic process or impairment of compensatory mechanisms. On several such occasions haemoglobin levels, usually stable at 8–9 g/dl fell as low as 4 g/dl, necessitating blood transfusion.

One individual (III 7) underwent splenectomy at age 25 years. He was the most symptomatic member of the family and had required hospitalisation and transfusion on a number of occasions. The mean of numerous pre-splenectomy haemoglobin determinations was 7.1 g/dl. At operation the spleen weighed 800 g. In the steady state following splenectomy (between the 5th and 12 months) his haemoglobin level has ever

aged 12.8 g/dl. Symptomatically he seems much improved and is functioning physically at a higher level.

Discussion

In other reports of families with unstable haemoglobins it has been suggested that there may be variable expression of the abnormal gene. Certainly in the present family the 8 affected members show a fairly consistent haematological picture with the possible exception of the oldest member (II 2) who appears less affected or has achieved better compensation. Many factors influence an individual's response to an undesirable gene such as that responsible for Hb Genova. These include the compensatory capacity of the bone marrow the functioning of the reticulo-endothelial system, the tolerance of the tissues to hypoxaemia, and the oxygen affinity of the abnormal haemoglobin.

Splenectomy has frequently been performed on patients with unstable haemoglobins, with variable results. In contemplating this procedure the possible advantages must be weighed against the hazards of the asplenic state and the extra burden imposed on the liver Our splenectomized patient (III 7) is perhaps the first case with Hb Genova in whom the diagnosis was known *pre-operatively and who has been* followed closely both before and after splenectomy This evaluation permits the conclusion that removal of the spleen, in this case at least, has been highly beneficial.

At least 10 different haemoglobin variants have been found in the indigenous population of Kenya [4 *Kendall* to be published]. Most of these have occurred in the Nilotic Luo and western Bantu Abaluhya populations. To date there have been virtually no variants detected in the central Bantu Kikuyu tribe, the most numerous population group in the country Thus the finding of the rare variant Hb Genova in a pure Kikuyu family is of special interest. In the 4 European families with Hb Genova, the origins have been Italian, French and Ukrainian. Based on family studies one individual may have acquired the anomalous gene as a new mutation [10] Almost certainly Hb Genova in our Kenyan family represents an independent mutation.

References

1 Betke, K., Marti, H. R., and Schlicht, L. Estimation of small percentages of foetal haemoglobin. Nature, Lond. *184* 1877–1878 (1959).

2 Carrell, R. W and Kay R. A simple method for the detection of unstable haemoglobins. Br J Haemat. *23* 615–619 (1972).

3 Dacie, J V Grimes, A. J Meisler A., Steingold, L., Hepsted, E. H., Beaven, G. H., and White, J C. Hereditary Heinz-body anaemia. A report of studies on five patients with mild anaemia. Br J Haemat. *10* 388–402 (1964).

4 Kendall, A G and Barr R. D Haemoglobinopathies in Kenya. Trans R. Soc. trop. Med Hyg. *67* 770–781 (1973).

5 Labie, D Bernadou, A Wajcman, H. et Bilski-Pasquier G Observation familiale d'une hémoglobine Genova β28(B10) Leu→Pro. Etude clinique, hématologique, génétique et biochimique d'une famille française. Nouv Revue fr Hémat. *12*. 502 506 (1977).

6 Sansone, G. and Pik, C. Familial haemolytic anaemia with erythrocyte inclusion bodies, bilifuscinuria and abnormal haemoglobin (Haemoglobin *Galliera Genova*). Br J Haemat. *11* 511–517 (1965).

7 Sansone, G Carrell, R. W and Lehmann, H. Haemoglobin Genova. β28(B10) Leucine→proline. Nature, Lond. *214* 877–879 (1967).

8 Sick, K. Beale, D. Irvine, D. Lehmann, H. Goodall, P. T. and MacDougall, S.: Haemoglobin G Copenhagen and haemoglobin J Cambridge. Two new β-chain variants of haemoglobin A. Biochim. biophys. Acta *140*: 231–242 (1967).

9 Singer, K. Chernoff, A. I. and Singer, L.: Studies on abnormal hemoglobins. I. Their demonstration in sickle cell anemia and other hematologic disorders by means of alkali denaturation. Blood *6*: 413–428 (1951).

10 Solal, M. C. and Labie, D. A new case of hemoglobin Genova $\alpha_2\beta_2$ (B [illegible]) Leu→Pro. Further studies on the mechanism of instability and defective synthesis. Biochem. biophys. Acta *295*: 67–76 (1973).

11 Wrightstone, R. N. Wilson, J. B. Reynolds, C. A. Huisman, T. H. J., Padmanabh, S., and Vella, F. Hb-Genova ($\alpha_2\beta$ [illegible](B [illegible]) Leu→Pro), methods for detection and analysis of unstable hemoglobins. Clinica chim. Acta *44*: 217–227 (1973).

Received: August 8, 1978
Accepted: October 18, 1978

Prof. H. Lehmann, FRS,
University Department of Biochemistry
Tennis Court Road,
Cambridge CB2 1QW (England)

Acta haemat. 61 283–287 (1979)

Reticuloendothelial Phagocytic Function in Children with β-Thalassemia Major

G Hicsönmez, E. Dalat and G Erbengi

Department of Pediatrics and Nuclear Medicine, Hacettepe University Children's Medical Center Ankara

Key Words. Reticuloendothelial phagocytic function β-Thalassemia major Children

Abstract. Reticuloendothelial phagocytic capacity (REPC) was determined in 14 children with β-thalassemia major by means of technetium 99m sulfur colloid uptake, who had not had splenectomy No difference was observed in the REPC between patient and controls. The REPC of liver and spleen were evaluated separately by determining the half-time for the clearance of ^{99m}Tc from the blood. The REPC of both liver and spleen in patients was expected to be decreased when compared with controls however we found that the REPC of the liver was increased in patients and that there was no difference between patients and controls spleen values. This suggests that chronic anemia and hemosiderosis do not alter the REPC in β-thalassemia major

It is widely believed that increased susceptibility to infection is one of the major complications in hereditary hemolytic anemias [6, 13] Patients with sickle cell anemia are known to have frequent and severe bacterial infections and several abnormalities in the body defense mechanisms have been reported [7 15–18, 23] However this is not well documented in patients with β-thalassemia major Chronic anemia and iron overload in reticuloendothelial system (RES) might cause decreased phagocytic capacity in those patients, but there has been no reported investigation concerning this subject. The present study was undertaken to evaluate the reticuloendothelial phagocytic capacity (REPC) in children with homozygous β-thalassemia.

Material and Methods

14 patients, 8 girls and 6 boys, with the age range of 4–20 years with β-thalassemia major who had not had splenectomy were studied. None of the patients had an infection at the time of the study The children and both parents were investigated hematologically for the diagnosis of β-thalassemia major On the same day that the patients were studied, 12 healthy children who did not have anemia and were in the same age group were studied as controls

Hb, Hct, and reticulocyte values were determined using standard techniques. Starch- and agar-gel electrophoreses were performed according t the method of *Smithies* [21], and *Robinson and Watson* [16], respectively Fetal Hb also was determined by the alkali denaturation method of *Singer et al.* [22], and quantitative estimation of Hb A_2 was obtained by *chromatography on diethylaminoethyl* cell lose (DEAE) [10] Incubated

erythrocyte osmotic resistance was carried out by the method of *Dacie and Lewis* [2]

Technetium 99m-sulfur colloid (^{99m}Tc) prepared according to the method of *Harper et al.* [9] was used to determined the REPC. A renaultron dual probe system was utilized for this purpose. The patient assumed the supine position and probes were placed on the spleen and the liver ^{99m}Tc was injected into the antecubital vein, the dosage ranged between 0.5 and 2 mCi according to the age of the patient. 2 min after the administration of ^{99m}Tc, 2-ml blood samples were drawn into heparinized tubes from the antecubital vein of the opposite arm, and repeated at 2-min intervals during the next 8 min. The plasma radioactivity was counted in a well type Nuclear-Chicago autogamma counter REPC was measured by the blood clearance of ^{99m}Tc and expressed as the phagocytic index (K) of RES which was calculated using the formula below [8]

$$K = \frac{\log C_1 - \log C_2}{T_2 - T_1}$$

C_1 and C_2 are the blood concentrations of test colloid at times T_1 and T_2 respectively

From the time of ^{99m}Tc injection the uptake of the radioactive material by the liver and the spleen was plotted on semilogarithmic paper until the plateau was obtained The half-time (T/2) of clearance of radioactive material by liver and spleen was calculated from the curve (fig. 1). The decrease in T/2 was indicative of an increase in clearance capacity of radioactive material by these organs.

Results

Some of the clinical data – number of the transfusions previous to the investigation and reticuloendothelial phagocytic index of patients – are shown in table I

The mean value of reticuloendothelial phagocytic index of patients with β-thalassemia major (0 112 ± 0 02) was not found to be statistically significant when compared with control values (0 113 ± 0.23) (table II)

The T/2 for the clearance of the radioactive material from the blood by liver and spleen There was no *statistically* significant difference in the T/2 of clearance of radioactive material by liver or spleen both in

Table I. Clinical data and reticuloendothelial phagocytic index (K) in patients with homozygous β-thalassemia

No	Age, years	Sex	Size of spleen cm	Size of liver cm	Number of transfusions	K value
1	4	F	15	8	1	0.09
2	4	F	22	5		0 04
3	5	F	9	3	1	0.13
4	5	M	2	4	–	0 09
5	7	F	1	3	5	0.12
6	7	F	9	4	3	0.10
7	8	F	14	5	6	0.09
8	10	F	14	7	60	0 12
9	10	M	18	9	4	0.11
10	11	M	18	10	52	0.09
11	12	M	20	16	5	0.11
12	14	F	10	5	–	0 10
13	16	F	10		–	0.17
14	20	M	7	5	3	0.13

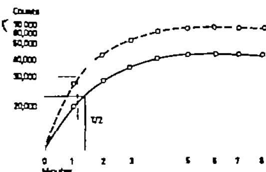


Fig. 1. 99mT clearance by the liver (○—○) and spleen (○——○).

Table II. Reticuloendothelial phagocytic index (K) in patients with homozygous β-thalassemia and controls

	Patients K value	Controls K value	
Mean ± SD	0.112 ± 0.02	0.113 ± 0.23	p > 0.05
Range	0.04 – 0.17	0.08 – 0.16	

Table III. The T/2 of the clearance of 99mT in patients with homozygous β-thalassemia and controls by liver and spleen

	Patients T/2 min	Controls T/2 min	
Spleen	0.96 ± 0.43	1.62 ± 1.45	p > 0.05
Liver	1.10 ± 0.39	1.53 ± 0.68	p < 0.05

Each value represents the mean ± SD in 14 patients and 12 controls.

The difference between the spleen and the liver values neither in patients nor in controls is statistically significant (p > 0.05).

patients and controls, also there was no significant difference between patients and controls' spleen values. However there was a significant decrease in the T/2 clearance value of the liver in patients with β-thalassemia major when compared with controls ($p < 0.05$) (table III).

Discussion

Although the susceptibility to infections in β-thalassemia major is not well documented, septicemia, and meningitis due to *Escherichia coli, Salmonella, Pneumococcus* and *Haemophilus influenzae* have been reported after splenectomy [3, 19]. However *Engelhard et al.* [4] showed that splenectomy is not responsible for the postsplenectomy infections. They suggested that some other factors might be the causes of these infections. Since ineffective erythropoiesis, chronic hemolysis, and frequent blood transfusions will be the cause of iron accumulation in RES in this disease, alteration of phagocytic function of these systems might be expected. However in the present study no difference was observed in the REPC of our patients and controls (table II). And also REPC of our patients was not correlated with the age, sex, size of liver and spleen, or the number of blood transfusions. Although in this study phagocytic capacity was determined using 99mTc-sulfur colloid, the possibility of deterioration of radioactive material was eliminated by studying the patients and controls in the same day.

Since the liver and the spleen are the major organs in iron overload we studied the phagocytic capacity of these organs separately by the determination of the T/2 for the clearance of 99mTc from the blood. No difference was found between the liver and spleen values in children with β-thalassemia major. Although the T/2 of clearance capacity of spleen did not show significant dif

ference from controls, increased clearance capacity of liver was observed when compared with controls (table III) The T/2 for the clearance of ^{99m}Tc from the blood by the liver and spleen did not show any correlation with the age and size of these organs. *Cooksley et al* [1] *did not find any relationship* between REPC and the spleen size in patients with cirrhosis, however they found that the correlation depended on the etiology *of the disease*

Increased clearance capacity of the liver in our patients might be explained by the hyperplasia of this organ and/or the smaller *amount of iron deposition in the liver than* in the spleen as was shown previously [14] *Jandl et al* [11] showed experimentally that hemolysis produced a proliferative response of *reticuloendothelial* cells. The hemolysis in children with β-thalassemia major may cause some increase in clearance capacity of the liver

The results of this study indicate that children with β-thalassemia major have normal REPC and the uptake of radioactive material from blood by the spleen and liver was not found decreased when compared with controls. This suggests that chronic anemia and hemosiderosis do not alter the phagocytic capacity of RES in this disease.

Acknowledgements

The authors wish to thank Dr *S Ozsoylu* for kindly reviewing this manuscript. We are also grateful to *Meeret Toprakci* for her technical assistance.

References

1 Cooksley W G E Powell, L W and Halliday J W Reticuloendothelial phagocytic function in human liver disease and its relationship to haemolysis. Br J Haemat. *25* 147–164 (1973).

2 Dacie, J V and Lewis, S. M Practical haematology. 3rd ed., pp 135–141 (Churchill, London 1963).

3 Diamond, C. K. Splenectomy in childhood *and the hazard of overwhelming infection. Pe*diatrics *43* 886–889 (1969).

4 Engelhard, D., Cividall, G and Rachmilewitz, E. A. Splenectomy in homozygous beta-thalassemia. A retrospective study of 30 patients Br J Haemat. *31* 391–403 (1975).

5 Eraklis, A. J and Filler R. M. Splenectomy in childhood. a review of 1413 cases. J pediat. Surg. 7 382–388 (1972).

6 Evans, H. E. and Reindorf, C. Serum immunoglobulin levels in sickle cell disease and thalassemia major Am. J Dis. Child. *116* 586–590 (1968).

7 Griesemer D A., Winkelstein, J A., and Luddy R. *Pneumococcal meningitis* in patients with a major sickle hemoglobinopathy J Pediat. *92* 82–84 (1978)

8 Groch, G S., Perillie, P E., and Finch, S. C. Reticuloendothelial phagocytic function in patients with leukemia, lymphoma, and *multiple* myeloma. Blood *26*. 489–498 (1965).

9 Harper H. V., Lathrop, K. A., Jiminez, F Fink, R., and Gottschalk, A. Technetium 99m as liver scanning agent. Radiology *85* 101–109 (1965)

10 Huisman, T H. J and Dozy A. M Quantitative determination of the minor hemoglobin component HbA_2 by DEAE-cellulose chromatography Ann. Biochem. 2. 400–403 (1961).

11 Jandl, J H. Files, N M., Barnett, S. B., and MacDonald, R. A. Proliferative response of the spleen and liver to hemolysis. J exp. Med. *122* 299–325 (1965).

12 Johnston, R. B Newman, S. L., and Struth, A. G. Serum opsonins and the alternate pathway in sickle cell disease. New Engl. J Med. *288* 803–808 (1973)

13 Nathan, D G and Oski, F A. Hematology of infancy and childhood, *pp 471–475* (Saunders, London 1974)

14 Okon, E., Levij, I. S., and Rachmilewitz, E. A. Splenectomy iron overload and liver cir

rhosis in β-thalassemia major Acta. haemat. 56. 142 150 (1976).

15 Pearson, H. A.; Spencer R. P and Cornelius, E. A.. Functional asplenia in sickle cell anemia. New Engl. J Med. *281* 923–926 (1969).

16 Robinson, M. G and Watson, R. J Pneumococcal meningitis in sickle cell anemia. New Engl. J Med. *274* 1006–1008 (1966).

17 Rosen, F S.. Sickle cell disease and the properdin system. New Engl. J Med. *283* 845–846 (1973).

18 Rubin, H. M., Eardley W and Nichols, B. C. *Shigella sonnei* osteomyelitis and sickle cell anemia. Am. J Dis. Child. *116*. 83–87 (1968).

19 Smith, C. H., Erlandson, M. E., Stern, G and Hilgartner, M. W Postsplenectomy infection in Cooley's anemia. New Engl. J Med. *266* 737–743 (1962).

20 Smith, C. H., Erlandson, M. E., Stern, G., and Hilgartner M. W Postsplenectomy infection in Cooley's anemia. Ann. N. Y Acad. Sci. *119*· 748–757 (1969).

21 Smithies, O.. Zone electrophoresis in starch gels. Group variations in the serum proteins of normal human adults. Biochem. J *61* 629–641 (1955).

22 Singer K., Chernoff, A. I., and Singer L.. Studies on abnormal haemoglobins. I. Their demonstration in sickle cell anemia and other hematologic disorders by means of alkali denaturation. Blood *6*. 413–428 (1951).

23 Winkelstein, J A. and Drachman, R. H.. Deficiency of pneumococcal serum opsonizing activity in sickle cell disease. New Engl. J Med. *279* 459–466 (1968).

Received: October 2, 1978
Accepted: November 17 1978

G. Hicsonmez, Department of Pediatrics, Hacettepe University Children's Medical Center Ankara (Turkey)

Acta haemat. 61 288–294 (1979)

Identification of an Erythrocyte Pyruvate Kinase Variant in a Family from Latium with Non-Spherocytic Congenital Haemolytic Anaemia

G Papa A De Laurenzi G C Isacchi G Bonifazi L. Parziale and A M Salvati

Cattedra di Ematologia, Università degli studi di Roma, and Istituto Superiore di Sanità, Lab Patologia non Infettiva, Roma

Key Words. Congenital non-spherocytic haemolytic anaemia Enzyme erythropathy Erythrocyte enzymes Red cell PK PK deficiency Mutant erythrocyte PK

Abstract. Erythrocyte PK deficiency was detected in a family from Latium in Italy This PK variant is characterized by normal or increased activity immediately after blood collection, instability to storage, to heat and to urea. Only in the propositus the mutant enzyme exhibited an increased Michaelis constant for PEP slightly increased inhibition by ATP and an altered optimum pH value The kinetic anomaly was only partially corrected by activation with F-1 6-DP and by addition of 2 ME.

From these results it can be concluded that in the family observed two distinct erythrocyte PK alterations were demonstrable instability in the propositus and his father low affinity for PEP and altered optimum pH value only in the propositus.

Valentine et al [22] described the first example of congenital non-spherocytic haemolytic anemia (CNSHA) due to deficiency of erythrocyte pyruvate kinase (PK)

Further investigations showed that in most cases the enzyme deficiency is due to structural modifications rather than to diminished enzyme synthesis. Numerous isoenzymes have been identified on the basis of alterations of kinetic and immunologic properties, stability pH optimum and electrophoretic mobility [6 7 12, 20 23]

In this paper we describe the physicochemical and kinetic abnormalities of erythrocyte PK observed in a family with CNSHA, from Latium.

Materials and Methods

Enzymes and substrates were supplied by Boehringer Mannheim and Sigma, Sephadex G-25 was supplied by Pharmacia and other reagents by Merck and C. Erba. Enzyme assays and kinetics were performed on a Gilford 2000 recording spectrophotometer Venous blood was anticoagulated with heparin or ACD and leukocytes filtered off on cotton [4]. Red cells were washed with buffered saline (Tris-HCl 17 m*M* NaCl 153 m*M* pH 7 4).

PK activity was determined according to the method recommended by the International Committee for Standardization in Haematology [4]. The results are expressed in international units (IU) per g of Hb. Other enzyme activities were measured according to previously published methods [10] and the values are expressed in IU per 10^{4} red blood cells (RBC).

Metabolic intermediates of RBC were determined according to *Beutler* [3] and *Bergmeyer* [2]. Pyruvate (PYR) concentration is expressed in nM per ml of blood, other metabolites in nM per ml of RBC. PK stability to heat and urea was tested according to *Miwa et al.* [12] and *Blume et al.* [5]. Optimum pH was determined in triethanolamine buffer (TRA 50 mM, EDTA 5 mM, KCl 100 mM, $MgCl_2$ 101 mM).

Kinetic characteristics were controlled according to *Beutler et al.* [6]. Tests were performed in TRA buffer pH 7.5 on the haemolysate previously filtered on Sephadex G-25 according to *Blume et al.* [5]. The reaction mixture for the determination of $K_{m\ app}$ for phosphoenolpyruvate (PEP) was the following: ADP 0.4 mM, NADH 0.2 mM, LDH 6 IU/ml, haemolysate 25–50 µl, PEP in progressive dilution from 1 to 0.05 mM. ATP inhibition was studied in a similar reaction mixture with two different tests: at constant ATP concentration (1.25 mM) with PEP varying from 0.1 to 1 mM and constant PEP concentration (0.25 mM) with ATP varying from 0.1 to 1 mM. Determination of $K_{m\ app}$ for PEP and inhibition tests with ATP were performed also in presence of fructose-1,6-diphosphate (FDP) 0.35 mM and after incubation for 1 h with 2-mercaptoethanol (2-ME) 10 mM according to *Van Berkel et al.* [24].

Case Reports

L. Ba. This 28-year-old man, from Villalba di Guidonia (Rome), had a history of subicterus since childhood. Physical examination demonstrated subicterus and a moderate enlargement of liver and spleen. Important blood data are reported in table I. ^{51}Cr red cell half-life was 29 days (normal values 27 ± 3 days) with splenic and hepatic sequestration within limits. No red cell autoantibodies were present. Abnormal haemoglobins could not be demonstrated, while Hb A_2 and Hb F levels were normal. Heinz bodies absent. Glutathione stability test [3] was negative. Chromosome analysis [14] disclosed no anomalies. Autohaemolysis test demonstrated a moderate increase of spontaneous haemolysis, up to 6.1% (normal values 0.4–1.5%), partially corrected by addition either of glucose (2.1%) or ATP (2.6%). 1 year later serum bilirubin persisted and increased, while red cell survival time was still normal.

L. Bl. This 67-year-old man was the propositus father. At the age of 60, cholecystectomy was performed for gallstones. Physical examination demonstrated subicterus and a moderate enlargement of spleen and liver. Important blood data are summarized in table I. Abnormal haemoglobins absent. Hb A_2 and Hb F levels were normal. Tests for red cell autoantibodies were negative. No increase of spontaneous haemolysis at autohaemolysis test. No chromosome abnormalities. No Heinz bodies could be demonstrated in red cells.

Other available members of the family were screened for haematological abnormalities (table

Table I. Clinical and laboratory data

	L.Ba. (propositus)	L.Bl. (father)	L.An. (sister)	L.Mad. (sister)	L.Mar. (sister)
Age, years	28	67	22	30	38
Hb, g/dl	12	14.2	11.3	12.3	12.9
PCV, %	40	40	38	40	43
RBC, 10^{6}/µl	4.2	4.2	4.0	4.2	4.6
MCH, pg	28.5	33.8	28.2	29.2	28
MCHC, %	30	35.5	29.7	30.7	30
WBC, 10^{3}/µl	6.5	5.4	4.6	6.2	6.2
Reticulocytes, %	7.2	6.0	0.8	4.2	2.0
Platelets, 10^{3}/µl	190	230	210	260	190
Serum iron, g/dl	120	80	120	75	75
Bilirubin, mg/dl					
Total	3.60	0.80	1.08	0.34	0.54
Direct	0.24	0.15	0.24	0.15	0.12

Acta haemat. *61* 288–294 (1979)

Identification of an Erythrocyte Pyruvate Kinase Variant in a Family from Latium with Non-Spherocytic Congenital Haemolytic Anaemia

G Papa A De Laurenzi G C Isacchi G Bonifazi L. Parziale and A M Salvati

Cattedra di Ematologia, Università degli studi di Roma, and Istituto Superiore di Sanità, Lab. Patologia non Infettiva, Roma

Key Words. Congenital non-spherocytic haemolytic anaemia Enzyme erythropathy Erythrocyte enzymes Red cell PK PK deficiency Mutant erythrocyte PK

Abstract. Erythrocyte PK deficiency was detected in a family from Latium in Italy This PK variant is characterized by normal or increased activity immediately after blood collection instability to storage, to heat and to urea. Only in the propositus the mutant enzyme exhibited an increased Michaelis constant for PEP slightly increased inhibition by ATP and an altered optimum pH value. The kinetic anomaly was only partially corrected by activation with F 1 6-DP and by addition of 2 ME.

From these results it can be concluded that in the family observed two distinct erythrocyte PK alterations were demonstrable· instability in the propositus and his father· low affinity for PEP and altered optimum pH value only in the propositus

Valentine et al [22] described the first example of congenital non-spherocytic haemolytic anemia (CNSHA) due to deficiency of erythrocyte pyruvate kinase (PK)

Further investigations showed that in most cases the enzyme deficiency is due to structural modifications rather than to diminished enzyme synthesis. Numerous isoenzymes have been identified on the basis of alterations of kinetic and immunologic properties, stability pH optimum and electrophoretic mobility [6 7 12, 20 23]

In this paper we describe the physicochemical and kinetic abnormalities of erythrocyte PK observed in a family with CNSHA from Latium.

Materials and Methods

Enzymes and substrates were supplied by Boehringer Mannheim and Sigma Sephadex G-25 was supplied by Pharmacia and other reagents by Merck and C. Erba. Enzyme assays and kinetics were performed on a Gilford 2000 recording spectrophotometer Venous blood was anticoagulated with heparin or ACD and leukocytes filtered off on cotton [4]. Red cells were washed with buffered saline (Tris-HCl 17 m*M* NaCl 153 m*M* pH 7 4).

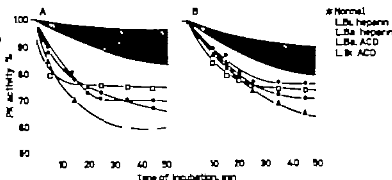


Fig. 1. Urea stability (A) and thermostability (B) tests of erythrocyte PK.

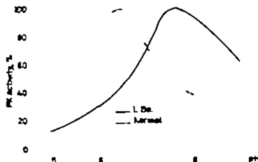


Fig. 2. Effect of pH on erythrocyte PK activity

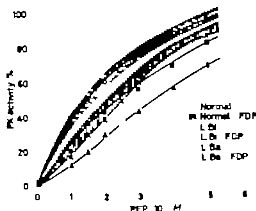


Fig. 3. Erythrocyte PK kinetic curves for PEP

values of $K_{m\ 0.5}$ *PEP* obtained from assays done at different times, are nearly twice normal with and without FDP after incubation with 2 ME they are reduced but not completely corrected (table IV). The values of Hill coefficient (n) were slightly higher than normal (table IV).

In the propositus ATP inhibition is increased. At constant PEP concentration (0.25 m*M*) and low ATP (up to 1 m*M*) the inhibition curve is shifted but presents a normal feature by adding FDP and 2 ME it is not completely corrected (fig. 4) At higher ATP concentrations (more than 1 m*M*) the difference from normal is not so evident. At constant ATP (1.25 m*M*) the activity curve against PEP concentration shows an abnormal feature within 0.2 and 0.5 m*M* PEP (fig. 5)

Discussion

The 2 cases observed of CNSHA are characterized by physico-chemical and kinetic abnormalities of erythrocyte PK. The father of the propositus had no anaemia and no hyperbilirubinaemia. An increased num-

I). Two sisters (L. An and L. Cr.) of the propositus gave a history of recurrent subicterus, one of them (L. Cr) had gallstones. Two other sisters are in good health. No information is available concerning the propositus mother who died of old age.

Results

PK activity of blood samples anticoagulated with heparin, within 2 h after blood collection, was reduced to about 50% of normal values in the propositus, while it was increased of about 25% in the father (table II) When blood samples were collected in ACD and examined within 2 h, levels of PK activity were markedly increased in both the propositus (140%) and his father (200%)

Enzyme activity was strongly reduced after 24 h storage at 4 C in both types of anticoagulants (table II) Control samples exhibited only a slight reduction. Other enzyme activities of the propositus were increased, especially aldolase, enolase and lactate dehydrogenase (table III)

The values of metabolic intermediates are reported in table III metabolites of the glycolytic pathway preceding PK (2,3-DPG and PEP) were increased as well as ADP and AMP On the contrary ATP pyruvate and GSH were reduced. ATP and PEP demonstrated the higher alterations. In the propositus father ATP was only slightly decreased and 2,3-DPG was normal.

PK was unstable in the propositus and his father and the curves of inactivation by heat and urea were markedly biphasic (fig. 1) The pH curve presented, in the propositus, a peak at 7.5 (fig. 2). The kinetic characteristics of erythrocyte PK, in the father were normal (fig. 3, table IV) In the propositus the enzyme showed a reduced affinity for PEP By addition of FDP PK activity was increased but the affinity curve was still abnormal (fig. 3) The average

Table II. Erythrocyte PK activity (IU/g of Hb) and stability in different anticoagulants

	Heparin		ACD	
	2 h	24 h	2 h	24 h
L.Ba. (propositus)	10.0	6.0	25 4	10.2
L.Bl. (father)	22.8	9.3	33.6	11 6
L.An. (sister)	–	5.0	–	–
20 normal controls	18.0 ± 3 0	17.2 ± 2.6	18.3 ± 3.2	18.1 ± .7

Table III. Enzyme activities and metabolic intermediates

	L.Ba. (propositus)	Normal controls
Enzymes, IU/10^{10} RBC		
HX	0.120	0.091 ± 0.025
PFK	1 750	1.328 ± 0.233
Aldolase	0.750	0.493 ± 0.107
TPI	95 400	64.532 ± 9 619
GADP	8.500	6.544 ± 1.122
PGM (+ 2,3 DPG)	8.000	6.625 ± 1.029
(PGM (– 2,3 DPG)	4.100	2.611 ± 0.754
Enolase	4.500	2.719 ± 0.624
LDH	51.800	31.039 ± 4.611
G6PD	1.350	1.074 ± 0.289
NADH-GR	0.390	0.301 ± 0.114
NADPH-GR	0.850	0.957 ± 0.122
Metabolites, nM/ml RBC		
ATP	680	1,200 ± 100
ADP	217	158 ± 15
AMP	64	50 ± 10
GSH	1 491	2,200 ± 200
PEP	122	20 ± 5
2,3-DPG	5,130	4,300 ± 200
PYR (nM/ml whole blood)	44	72 ± 14

2-ME, and the increased inhibition by ATP point out a molecular alteration specifically affecting the affinity for PEP which does not depend on the presence of altered thiol groups, as reported by *Van Berkel et al* [24] in other PK mutants.

These alterations bring about a reduction of glycolysis, as shown by the increase of metabolites preceding PK in the glycolytic pathway and the reduced level of ATP Glutathione is also reduced. this fact cannot be directly related to the enzyme deficiency: it could be explained on the basis of the interactions between the glycolytic pathway the pentose shunt and the redox systems of red cell, as demonstrated by others [1 8 18,19]

The increased activity of other glycolytic enzymes (table III) could depend on the reticulocytosis and the presence of a younger erythrocyte population. Our variant could belong to the third group described by *Miwa et al.* [12] The excess of FDP does not correct $K_{m\ app}$ PEP and one might suppose that, according to the R⇄T model [13], there is no balance shift towards T as described for other variants [9 21]

PK deficiencies described by *Van Berkel et al.* [24] and *Zanella et al.* [25] were corrected by 2 ME, this has suggested that the enzyme deficiency could depend on a primary unknown defect involving red cell thiol groups. This is not true in our case, since 2 ME does not cause an appreciable increase of enzyme affinity for substrate.

Kinetic alterations of PK are present in the propositus only while spontaneous instability and lability to physical and chemical agents have been demonstrated in both propositus and his father As it was impossible to examine the propositus mother it can only be supposed that in this family there are two different alterations of the enzyme molecule, an increased lability and a diminished affinity for the substrate.

In conclusion, the PK of the propositus is characterized by thermal, urea and storage lability an optimum pH at about 7.5 an increased K_m for PEP which is not corrected by FDP and 2 ME and an ATP inhibition higher than normal.

The variant described here thus appears to differ from those previously described and it is proposed to call it PK Latium.

References

1 Almar, F Arese, P Bosia, A., Gaetani, G. F Pescarmona, G. P.: Correlazione fra attività enzimatica ed emolisi nelle anemie emolitiche non sferocitiche. Proc. 6th Int. Symp. on Clin. Enz., pp. 77–92 (Burlina, 1974).

2 Bergmeyer H. U Methods of enzymatic analysis 1432, 1446; 1643 2101, 2131 (Verlag Chemie, Weinheim/Academic Press, New York 1974).

3 Beutler E. Red cell metabolism: manual of biochemical methods, p. 17 (Grune & Stratton, New York 1972)

4 Beutler E.; Blume, K. G Kaplan, C. J Lohr G W Ramot, B., and Valentine, W N. International Committee for Standardization in Haematology – Recommended methods for red cell enzyme analysis. Br. J Haemat. 35 331 340 (1977).

5 Blume, K. G., Arnold, H., Lohr G. W and Beutler E. Additional diagnostic procedure for the detection of abnormal red cell pyruvate kinase Clinica chim Acta 43 443–446 (1973).

6 Bolvin, P Galand, C. et Demartial, M. C., Coexistence de deux types de pyruvate kinase cinétiquement différents dans les globules rouges humains normaux. Nouv Revue fr Hémat. 12 159–169 (1972).

7 Boc, H Najman, A.; Columelli, S et Cartier D Déficit congénital en pyruvate kinase érythrocytaire: étude cinétique de l'enzyme et conséquences métaboliques. Clinica chim. Acta 38. 131 140 (1972).

Table IV Kinetic characteristics of erythrocyte PK

		L. Ba. (propositus)		L. Bl (father)		Normal controls	
		FDP(+)	FDP(−)	FDP(+)	FDP(−)	FDP(+)	FDP(−)
n of Hill		1.03	1.06	1.01	1.02	0.9–1.00	1.01–1.03
K_{m} PEP mM	2 ME(+)	0.14	0.26	–	–	0.07–0.10	0.12–0.20
	2 ME(−)	0.17	0.47	0.09	0.27	0.08–0.11	0.21–0.37

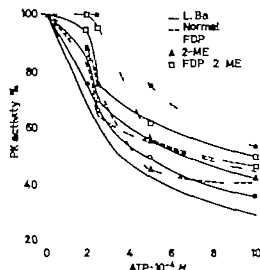


Fig. 4. Erythrocyte inhibition at constant PEP concentration by varying ATP concentration.

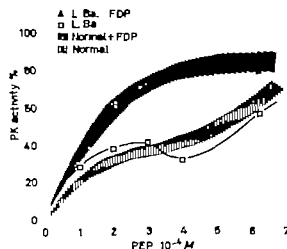


Fig. 5. ATP inhibition of erythrocyte PK by varying PEP concentration.

ber of reticulocytes is indicative of a compensated haemolysis. The same is true for one sister (L. Mad. in table I)

In the propositus ^{51}Cr red cell half-life was normal with no pathological sequestration in spleen and liver area. Other authors [11 15 16] have already noticed a discrepancy between RBC survival time and the presence of anaemia with increased reticulocytes and serum bilirubin. *Najean et al.* [15] demonstrated that some of the labelled cells are destroyed in the spleen and liver within the first minutes after reinjection, thus giving a false baseline for further measurements.

The red cell PK was more stable in ACD than in heparin anticoagulated samples this different behaviour has not been reported elsewhere. The enzyme stability in presence of denaturing agents (heat and urea) is markedly impaired these aspects are similar to those described by *Staal et al* [20]

The biphasic pattern of the inactivation curves of the blood samples collected in heparin suggests the existence of an enzyme fraction markedly unstable, also spontaneously unstable, although to a lesser degree. This biphasic pattern is not so evident in blood samples collected in ACD probably because of the correcting influence exercised by glucose.

The low affinity of PK for PEP which cannot be completely corrected by FDP and

Short Communications

Acta haemat. 61 295–297 (1979)

Differences between Individuals with Hemoglobins Istanbul and Saint-Etienne ($\alpha_2\beta_2$ 92F8 His→Gln)[1]

Muzaffer Aksoy and Sakir Erdem

Section of Hematology Internal Clinic of Istanbul Medical School, Çapa, Istanbul

Key Words. Hemoglobin Istanbul Fetal hemoglobin Splenectomy

Abstract. The unstable hemoglobins Istanbul and Saint Etienne have the same amino acid substitution ($\alpha_2\beta_2$92F8 His→Gln) Despite this, there are some clinical and hematological differences between the individual with Hb Istanbul and the one with Hb Saint Etienne. These are: (1) the clinical course of the patient with Hb Istanbul before splenectomy was more severe when compared with that of the individual with Hb Saint Etienne (2) although Hb F was found within normal range in the individual with Hb Istanbul, a high level of Hb F (19%) was observed in the patient with Hb Saint Etienne The alkali-resistant Hb found in the former was of adult type and in the latter case it was of fetal type The possible explanations for these differences are discussed.

The unstable hemoglobin Istanbul ($\alpha_2\beta_2$92F8 His→Gln) was found in a 29-year-old Turkish male [1] Hb Istanbul and Hb Saint-Etienne, which was found by *Rosa et al* [2] in a 8-year-old French boy have the same amino acid substitution. Despite this, there are some clinical and hematological differences between the individual with Hb Istanbul and the individual with Hb Saint-Etienne These differences are summarized in table I

The patient with Hb Istanbul had a moderate anemia and frank signs of increased hemolysis such as hyperbilirubinemia and reticulocytosis. The clinical picture and hematological findings ameliorated considerably after splenectomy Now 9 years after splenectomy the patient enjoys a very active normal life except some minor complaints. Hb rose from 9 1 to 11 6 g/dl and packed red cells from 32 to 40%. Reticulocytes dropped from 4.2 to 3 6% and total bilirubin from 3 to 1.2 mg/dl. Despite this, the persistence of ahaptoglobinemia and mild reticulocytosis is strong evidence of the presence of a compensated hemolytic process.

Another minor difference was the presence of a few intraerythrocytic Heinz bodies in the individual with Hb Istanbul whereas

[1] From the Unit of Hemolytic Anemias and Chemical Hematotoxic Disorders, supported by (TBTAK).

8 Glader B E. Oxidant induced injury in pyruvate kinase deficient erythrocytes. Pediat. Res. *8* 401–407 (1974).

9 Kahn, A. Vives Corron, J L. Marie, J., Galand, C., and Boivin, P.. A Spanish family with erythrocyte pyruvate kinase deficiency contribution of various immunologic methods in the study of the mutant enzyme. Clinica chim. Acta *75* 71–80 (1977).

10 Mandelli, F Deriu, L., De Laurenzi, A. Fontana, L. e Papa, G.. Comportamento di alcuni enzimi e metaboliti negli eritrociti dei soggetti normali. Rec. Prog Med *41* 205–231 (1966).

11 Mentzer W C., Jr Baehner R. L., Schmidt Schonbein, H. Robinson, S. H., and Nathan, D G Selective reticulocyte destruction in erythrocyte pyruvate kinase deficiency J clin. Invest. *50* 688–699 (1971).

12 Miwa, S. Nakashima, K., Ariyoshi, K. Shinohara, K., Oda, E., and Tanaka, T.. Four new pyruvate kinase variants and a classical PK deficiency Br J Haemat. *29* 157–169 (1975)

13 Monod, J., Wyman, J and Changeux, J P On the nature of allosteric transition. a plausible model. J molec. Biol. *12* 88–118 (1965)

14 Moorhead, P S. Nowell, P C., Mellman, W J Battips, D M., and Hungerford, D A.. Chromosome preparations of leukocytes cultures from human peripheral blood. Expl. Cell Res. *20* 613–616 (1960).

15 Najean, Y., Dresch, C. et Bussel, A.. Etude érythrocinétique dans 8 cas de déficit homozygote en pyruvate kinase. Nouv Revue fr Hémat. *9* 850–859 (1969).

16 Nathan, D G Oski, F A., Miller D R., and Gardner F H. Life span and organ sequestration of the red cells in pyruvate kinase deficiency New Engl J Med. *278* 73–81 (1968).

17 Paglia, D E., Valentine, W N., and Rucknagel, D L.. Defective erythrocyte pyruvate kinase with impaired kinetics and reduced optimal activity Br J Haemat. *22* 651–665 (1972)

18 Rose, I A. and Warms, J W B Control of red cell glycolysis. The cause of triose phosphate accumulation. J biol. Chem. *245* 4009–4015 (1970).

19 Srivastava, S. K. and Beutler E.. The effect of normal red cell constituents on the activities of red cell enzymes. Archs Biochem. Biophys *148* 249–255 (1972)

20 Staal, G E. J., Coster J E., and Nijssen, J. G A new variant of red blood cell pyruvate kinase deficiency Biochim. biophys. Acta *258* 685–687 (1972).

21 Staal, G E J Ceerdink, R. P Vlug, A M C., and Hamelink, M L.. Defective erythrocyte pyruvate kinase. Clinica chim. Acta *68* 11–15 (1976).

22 Valentine, W N Tanaka, K. R., and Miwa, S.. Specific glycolytic enzyme defect (pyruvate kinase) in three subjects with congenital nonspherocytic haemolytic anaemia. Trans Ass. Am. Physns *74* 100–110 (1961).

23 Valentine, W N.. Deficiencies associated with Embden-Meyerhof pathway and other metabolic pathways. Semin. Haemat. *8*. 348–366 (1971).

24 Van Berkel, T J C., Staal, G. E. J., Coster S. E., and Nijssen, J G.. On the molecular basis of pyruvate kinase deficiency Role of thiol groups in PK from PK deficient patients. Biochim. biophys. Acta *334* 361–367 (1974).

25 Zanella, A. Rebulla, P Giovannetti, A. M., Curzio M., Pescarmona, G P and Sirchia, G Effects of sulphydryl compounds on abnormal red cell pyruvate kinase. Br J Haemat. *32* 373–385 (1976).

Received. February 17 1978
Accepted October 8, 1978

Dr Giuseppe Papa, Cattedra di Ematologia, Università degli studi di Roma, Via Lancisi 3, I-00100 Roma (Italy)

References

1 Aksoy M Erdem, S., Efremov G. D., Wilson, J. B., Huisman, T H. J Schroeder W A., Shelton, J R., Shelton, J. B., Ulutin, O. N., and Müftüoğlu, A., Hemoglobin Istanbul. substitution of glutamine for histidine in proximal histidine (F8(92)β). J clin. Invest. 51 2380–2387 (1972).

2 Rosa, J Brizard, C. P Gibaud, A., Beuzard, Y., Courvalin, J C., Cohen-Solal, M., Carrel, M. C. et Thillet, J L'hémoglobine Saint Etienne: $\alpha_2\beta_2$ 92 His→Gln (F8), une nouvelle variété d'hémoglobine instable avec absence d'hème. Nouv Revue fr Hémat. 12 691–699 (1972).

3 Godeau, J F., Beuzard, Y C., Cacheleux, J Brizard, C. P Gibaud, A., and Rosa, J Association of hemoglobin Saint-Etienne ($\alpha_2\beta_2$92F8 His→Gln) with hemoglobins A and F Synthesis and subunit exchange *in vitro*. J biol. Chem. 251 4346–4354 (1976).

4 Wood, J. B., Clegg, J B., and Weatherall, J D Developmental biology of human haemoglobins. Prog. Hemat. 10· 43–90 (1977).

5 Aksoy M., Dinçol, G and Erdem, S. Sickle cell syndromes in Turkey Symposium on abnormal hemoglobins. Istanb. Contr clin. Sci. (in press)

Received. April 27 1978
Accepted: September 29 1978

Prof. Dr. Muzaffer Aksoy
Internal Clinic of Istanbul Medical School,
Capa, Istanbul (Turkey)

Table I. Differences between individuals with Hb Istanbul and Hb Saint-Etienne

Findings	Patient with Hb Istanbul		Patient with Hb Saint-Etienne
	before splenectomy	9 years after splenectomy	
Clinical course	moderate	mild	mild
Anemia	moderate	mild	mild
Spherocytes in peripheral smear	absent	some	absent
Intraerythrocytic inclusion bodies, %	1	50	0
Hb F %	2	2	19
Abnormal Hb fraction, %	12–15	15	26
The ratio of G Aγ		2 3	0.81.3
Genetic pattern	mutation		mutation

none were found in the patient with Hb Saint Etienne. Following splenectomy the percentage of Heinz bodies reached nearly 50% and also some spherocytes appeared in the peripheral blood smear of the patient with Hb Istanbul this was also observed in indviduals with other unstable hemoglobins following this surgical procedure

The important difference between the two individuals with Hb Istanbul and Hb Saint Etienne is the wide variation in the percentage of Hb F and the Gγ:Aγ ratio The *alkali resistant Hb* was within normal range in the patient with Hb Istanbul. By contrast, the percentage of Hb F in the individual with Hb Saint Etienne was markedly increased, 19% at the age of 11 years, even after 5 years of follow-up Recently this difference was discussed by *Godeau et al* [3] and *Wood et al.* [4] The alkali resistant Hb found in the patient with Hb Saint Etienne was of the fetal type [3] On the contrary the small amount of Hb F encountered in our patient with Hb Istanbul was of adult type [1]

How can one explain this difference in the level and also in the type of Hb F found in the individuals with Hb Istanbul and Hb Saint Etienne? The following possibilities may be considered in this respect.

(1) Contrary to the individual with Hb Istanbul, two mutations might have occurred in the patient with Hb Saint-Etienne. The first one altered the amino acid composition leading to the substitution of F892 His→Gln, similar to that of Hb Istanbul. The second mutation is in some way related to the increased level of Hb F

(2) The second mutation did not occur but some unknown factors might have been the cause of the increase in the level of Hb F This mechanism must be different from the one that operates in heredritary persistence of Hb F

On the other hand, variation in the level of Hb F is not uncommon in other hemoglobinopathies. For instance, in sickle cell anemia, the Hb F level varies considerably from patient to patient, from 0% to more than 20% [5] The exact nature of this variation is unknown. The question of which one of these possibilities is responsible for the elevation of Hb F in the individual with Hb Saint Etienne remains unanswered.

Maternity Hospital Dublin. Complete remission was confirmed 2 weeks after the first induction course. Three consolidation courses were given, separated by 21-day intervals. These consisted of the first 5 days of the induction regime already outlined.

Spontaneous vaginal delivery of a male infant occurred on 17th October 1977. Birth weight was 5 kg (glucose tolerance test of the mother was normal). The baby's full blood count and karyotype were normal, and he remains well 6 months after delivery. Having received CNS prophylaxis the mother remains in remission on daily 6-mercaptopurine and weekly cyclophosphamide.

Case II

A 24-year-old woman was referred on 28th July 1977 from the National Maternity Hospital Dublin for investigation of pancytopenia. She was in the 15th week of her first pregnancy and complained of nausea, vomiting and rigors which had developed 2 weeks previously. There was pallor but no evidence of bruising, petechiae or superficial lymphadenopathy. The splenic tip was palpable. Haemoglobin was 9.8 g/dl, platelets 95×10^9/l. White cell count was $1{,}000 \times 10^9$/l, with a differential of 48% polymorphs, 52% lymphocytes. No primitive forms were noted on blood film examination. On transfer to our unit, bone marrow aspirate was taken from the posterior iliac crest. A diagnosis of acute lymphoblastic leukaemia (ALL) was established.

Therapy was commenced as outlined in case I. Complete remission followed the first induction course within 2 weeks. Consolidation courses were subsequently given at 21-day intervals. The fetus was viable and satisfactory growth was monitored at the National Maternity Hospital Dublin. At 29 week's gestation severe pre-eclamptic toxaemia developed and responded poorly to the usual management. 1 week later intra-uterine death was confirmed by ultrasonography. A dead fetus was delivered 5 days later. No congenital abnormalities were noted at post-mortem examination. Unfortunately karotyping was unsuccessful. Symptoms of toxaemia rapidly subsided following delivery. CNS prophylaxis has been administered and at time of writing the patient has been in continuous remission for 6 months. She is on 6-mercaptopurine and cyclophosphamide maintenance.

Discussion

In 1968 *Nicholson* [5] reviewed 185 human pregnancies reported in the literature, in which cytotoxic drugs had been used. Acute leukaemia had been the indication for administration of such drugs in 42 cases. He concluded that a significant risk of fetal malformation was present following treatment which had commenced in the first trimester. The risk to the fetus appeared no greater than normal during the second and third trimesters.

Cytosine arabinoside has been more recently introduced. 5 cases have been described of its use in patients with acute myeloid leukaemia in pregnancy [3–7]. Four of these included thioguanine in the therapeutic regime. *Maurer et al.* [4] describe apparent fetal group C trisomy following chemotherapy incorporating cytosine arabinoside and thioguanine instituted during the 20th week of pregnancy. Delivery of normal infants following chemotherapy begun at times ranging from the 25th to 31st weeks of gestation is described by other authors [3, 5–7].

Our first case is of interest in that daunorubicin formed part of the chemotherapeutic regime. As far as we are aware, use of this drug has not been reported in a pregnant patient with acute leukaemia. We used the 'TAD' regime as described by *Gale and Cline* [3]. They achieved an overall response rate of 82% in a series of 28 patients with AML using a high-dose sequential remission-induction regime of cytosine arabinoside, 6-thioguanine and daunorubicin, as outlined in case I. Our patient achieved and has maintained remission on this regime and delivered a normal infant of high birth weight. *Nicholson* [5] reports an increased

Acta haemat. 61 298–300 (1979)

Two Cases of Acute Leukaemia in Pregnancy

Rory O Donnell Colm Costigan and Liam G O Connell

Department of Haematology St. Vincent's Hospital, Dublin

Key Words. Acute myeloblastic leukaemia Acute lymphoblastic leukaemia Chemotherapy in pregnancy Leukaemia in pregnancy

Abstract. 2 cases of acute leukaemia which developed in the course of pregnancy are reported The first was a 34-year-old woman who presented with acute myeloblastic leukaemia late in the second trimester and received combination chemotherapy A normal male infant was delivered. The second patient, aged 24 years, presented with acute lymphoblastic leukaemia early in the second trimester and was treated with the same regime. Pre-eclamptic toxaemia developed at 29 weeks gestation. Intra-uterine death was confirmed 1 week later

Introduction

Development of acute leukaemia during pregnancy is uncommon Therapy involving cytotoxic agents poses hazards to the fetus [2] However some recent reports of normal deliveries following intensive chemotherapy are encouraging [2, 5–7] We report two further contrasting cases.

Case Reports

Case I

A 34-year-old woman in her fourth pregnancy was admitted on 15th July 1977 She gave a 3-week history of increasing lassitude, dyspnoea on exertion and easy bruisability Physical examination revealed pallor scattered petechiae and bruising of the lower limbs. Haemoglobin was 5.8 g/dl white cell count 35 6 × 10^9/l, with a differential of 64%/ myeloblasts. Auer rods were prominent. Platelet count was 13.0 × 10^9/l. Cytology and cytochemistry of a bone marrow aspirate from the posterior iliac crest established a diagnosis of acute myeloblastic leukaemia (AML).

Length of gestation was estimated at 27 weeks. A single live fetus whose growth corresponded to gestational age was demonstrated by ultrasonography Therapy was commenced according to the 'TAD' routine described by *Gale and Cl[illegible] e* [3]: i.e. (I) thioguanine 100 mg/M^2 orally every 12 h on the 1st to 7th days (inclusive); (II) cytosine arabinoside 100 mg/M^2/intravenously over 30 min every 12 h on the 1st to 7th days (inclusive); (III) daunorubicin 60 mg/M^2/day on the 5th, 6th and 7th days. Allopurinol 100 mg t.i.d. was also given.

With the welfare of the now viable fetus in mind, management was continued at the National

Original Papers

Acta haemat. 61: 301–309 (1979)

Clonal Evolution of Marker Chromosomes in a Case of Myelofibrosis with Myeloid Metaplasia and Myeloblastic Transformation

Brenda M. Page, Jessie L. Watt, I. N. Reid, R. J. L. Davidson and W. Walker

Departments of Genetics and Pathology, Haematology Unit, Department of Pathology and Department of Therapeutics and Clinical Pharmacology, University of Aberdeen, Foresterhill, Aberdeen

Key Words. Banding · Bladder · Clonal evolution · Marker chromosomes · Myeloblastic transformation · Myelofibrosis · Neutrophil alkaline phosphatase

Abstract. The diverse spectrum of acquired chromosome abnormalities in a female patient with myelofibrosis and myeloid metaplasia is described. A sequence of karyotypic evolution involving a ring chromosome is postulated. The terminal clinical picture was unusual in that there was obstructive renal failure from extramedullary myeloblastic transformation and infiltration of the bladder, and this was also present in other sites. Initially neutrophils showed low alkaline phosphatase activity but latterly two distinct populations in which cells had either high activity or none.

Myelofibrosis with myeloid metaplasia is a chronic disease characterised by: splenomegaly, often with hepatomegaly; the presence in the peripheral blood of immature myeloid and erythroid elements and often large and bizarre platelets, anaemia with anisopoikilocytosis and frequent tear-drop forms; variable degrees of marrow fibrosis, extramedullary haematopoiesis, most marked in the spleen and the liver; normal or elevated neutrophil alkaline phosphatase activity; and absence of specific chromosomal aberrations [*Dameshek and Gunz*, 1964; *Glew et al.*, 1973; *Ward and Block*, 1971]. The leucocyte count is normal or more commonly increased to between 10 and 20×10^{9}/l, but counts up to 50×10^{9}/l are not infrequent, and an increase in basophils is usual.

In a recent cytogenetic survey of 150 of our patients with various blood disorders, 52 had myeloproliferative syndrome, and of these nearly half had an acquired chromosome abnormality. Of 19 patients with myelofibrosis only 5 showed karyotypic changes despite intensive and repeated investigation.

Our findings therefore confirm that, in myelofibrosis, chromosome abnormality is not common. In particular the classical Philadelphia (Ph¹) chromosome characteristic of chronic myeloid leukaemia (CML) is not present [*Cohen*, 1967]. Of our 5 pa-

frequency of low birth weight infants in patients receiving chemotherapy during the second and third trimesters

In contrast, our second patient had ALL and presented early in the second trimester It is well known that adults with ALL respond poorly to conventional therapy In an effort to achieve remission rapidly we tried the 'TAD regime as used in AML. Complete remission followed the induction course and has so far been maintained. It is unfortunately impossible to determine if the development of pre-eclamptic toxaemia in this primigravid patient, followed by delivery of a dead fetus was in any way related to chemotherapy or to the leukaemic process.

Acknowledgement

We would like to acknowledge the help of the Master and staff of the National Maternity Hospital Dublin for their cooperation in the management of these cases.

References

1 Au-Yong, R. *Collins, P* and Young, *J A.* Acute myeloblastic leukaemia during pregnancy Br med. J *iv* 493–494 (1972)

2 Durie, B. G M. and Giles, H R. Successful treatment of acute leukaemia during pregnancy Archs intern. Med. *137* 90–91 (1977).

3 Gale, R. P and Cline, M. J High remission-induction rate in acute myeloid leukaemia Lancet *i.* 497 (1977).

4 Maurer L. H. Forcier R. J., McIntyre, O R., *et al.*, Fetal group C trisomy after cytosine arabinoside and thioguanine. Ann. intern. Med. *75* 809–810 (1971)

5 Nicholson, H. O Cytotoxic drugs in pregnancy: review of reported cases. J Obstet. Gynaec. Br Commonw *75* 307–312 (1968).

6 Pawliger D F., McLean, F W and Noyes, *W D Normal foetus after cytosine* arabinoside therapy Ann. intern. Med. *74* 1012 (1971).

7 Raich, P C. and Curet, L. B., Treatment of acute leukaemia during pregnancy Cancer *36* 861–862 (1975)

Received. August 22, 1978
Accepted. November 15 1978

Dr Liam G O'Connell,
Consultant *Haematologist,*
St. Vincent's Hospital,
Elm Park, Dublin 4 (Ireland)



Fig. 1. Section of bone marrow showing fibrosis, reduction of normal haemopoietic elements and megakaryocyte hyperplasia. HE × 185. b Section of bladder wall showing infiltration by haemopoietic elements. Megakaryocyte in centre of field HE. × 185

Fig. 2. Q-banded bone marrow partial metaphases. Showing the Ph 1 ke marker associating at each telomere with 21 and 15 chromosome respectively. b Acrocentric marker adjacent to 15 chromosome to compare the former large size.

Fig. 3. Sequential Q- and C-banded partial karyotypes of markers combined in three different cells (A, B and C). Left. the t (1 2) markers with normal homologues, middle: ring marker right: submetacentric marker

tients with positive chromosomal findings 4 had relatively minor changes 3 had atypical Philadelphia chromosomes [*Watt et al* 1977] 1 having additional trisomy 8 1 had a clone of about 60 chromosomes affecting 20% of cells and 1 the subject of this report, had the broadest spectrum of chromosomal changes encountered in our patients with blood disorders.

Case Report

Mrs. E. C., aged 61 presented in November 1971 with tiredness and left hypochondrial pain. The only notable physical signs were a spleen enlarged half-way to the umbilicus and some oedema of the left leg. Examination of the peripheral blood showed Hb 11 g/dl, WBC 9.2 × 10⁹/l (neutrophils 67%, basophils 3%, lymphocytes 17%, monocytes 5%, metamyelocytes 4%, myelocytes 2%, myeloblasts 2%) normoblasts 3/100 white cells reticulocytes 3% platelets 160 × 10⁹/l. The blood film showed anisopoikilocytosis of the red cells with 'tear-drop forms platelets included abnormal giant forms and some megakaryocytic nuclear fragments were seen. Neutrophil alkaline phosphatase score was 8 (normal 35–100) Direct antiglobulin test was negative. Serum vitamin B was > 1,000 ng/l and folate 6.2 g/l Sternal marrow on two punctures was difficult to aspirate and hypocellular with normoblastic erythropoiesis and an increased number of primitive reticulum cells. Iliac crest marrow biopsy showed depression of all normal elements, large numbers of fibroblast like cells and an increase in megakaryocytes Cultures of marrow for chromosome analysis failed but no Ph chromosome was seen in peripheral blood. Chest X-ray was normal, blood urea 6 mmol/l and uric acid 0.18 mmol/l Other investigations including a range of auto-immune tests were negative. The diagnosis was atypical myelofibrosis with deep venous thrombosis in the left leg, all signs of the latter clearing with high elevation of the limb

Between 1971 and 1976 she remained generally well but with recurrent superficial phlebitis in both legs. The Hb level varied from 8 to 10 g/dl and reticulocytes from 3 to 10%, tending to rise. The leuco-erythroblastic blood picture persisted and the spleen enlarged beyond the umbilicus. In November 1972 the serum folate level was 2.4 μg/l and folic acid 10 mg daily was given. In April 1976 she had symptoms of hypothyroidism, serum thyroxine was under 20 nmol/l and TSH 54 μU/ml thyroxine was given, rising to a maintenance dose of 150 g daily At this time Hb was 7 g/dl and she had her first transfusion of packed red cells. In August 1976, Hb being 8 g/dl and reticulocytes 8%, prednisolone was started and continued in a dose of 2.5 mg three times daily though the mechanism of any haemolysis was almost certainly hypersplenic; the direct antiglobulin test remained negative or doubtfully positive. However prednisolone appeared to give symptomatic improvement and Hb rose again to 10 g/dl. In March 1977 serum urate was raised at 0.51 mmol/l.

In August 1977 she was admitted with left ilio-femoral vein thrombosis giving gross oedema which cleared with high elevation, bandaging and 6 days of intravenous heparin. Blood examination showed Hb 6 7 g/dl 11 6 g after transfusion, WBC 5.0 × 10⁹/l with 10% blast cells, and platelets 75 × 10⁹/l blood urea was 7.6 mmol/l, urate 0.51 mmol/l creatinine 98 μmol/l. On 3 October 1977 she was symptom-free but Hb was 7.8 g dl.

On 11 November 1977 she was readmitted with a history of dribbling urinary incontinence for 4 weeks, and probably oliguria which was obvious after admission, urinary output falling from 300 to less than 100 ml/day The urine contained both protein and blood. There was severe cardiac failure with gross generalised oedema, all of the signs of failure except the oedema improving after Hb was raised from 5 to 8 g/dl by transfusion of packed cells. At this time the WBC was 5 7 × 10 /l with 19% myeloblasts neutrophil alkaline phosphatase score was 129 with activity in only 47% of cells, the rest having very low or absent activity which suggested two populations of neutrophils. Platelets numbered 72 × 10⁹/l Sternal marrow gave inconclusive results but chromosome changes were grossly abnormal and had diagnostic implications. Blood urea rose from 36 to 70 mmol/l urate from 0.95 to 1.3 mmol/l, and creatinine was 814 μmol/l. Total serum protein was 64 g/l and albumin 30 g/l. An intravenous pyelogram gave no excretion and ultrasonic scanning showed bilateral hydronephrosis. She died on 19 November 1977



Fig. 1. Section of bone marrow showing fibrosis, reduction of normal haemopoetic elements and megakaryocyt hyperplasia. HE × 185 b Section of bladder wall showing infiltration by haemopoietic elements. Megakaryocyte in centre of field HE × 185.

Fig. 2. G-banded bone marrow partial metaphases Showing the Ph -hk marker associating at each telomere with 21 and 15 chromosome respectively b Acrocentric marker adjacent to 15 chromosome to compare the former's large size

Fig. 3. Sequential G- and C-banded partial karyotypes of markers combined in three different cells (A, B and C). Left: *the t (1, 2) markers with* normal homologues, middle ring marker right: submetacentric marker

tients with positive chromosomal findings 4 had relatively minor changes 3 had atypical Philadelphia chromosomes [*Watt et al* 1977] 1 having additional trisomy 8 1 had a clone of about 60 chromosomes affecting 20% of cells and 1 the subject of this report, had the broadest spectrum of chromosomal changes encountered in our patients with blood disorders.

Case Report

Mrs. E. C., aged 61 presented in November 1971 with tiredness and left hypochondrial pain The only notable physical signs were a spleen enlarged half way to the umbilicus and some oedema of the left leg. Examination of the peripheral blood showed Hb 11 g/dl, WBC 9.2×10^9/l (neutrophils 67%, basophils 3%, lymphocytes 17%, monocytes 5%, metamyelocytes 4%, myelocytes 2%, myeloblasts 2%) normoblasts 3/100 white cells, reticulocytes 3%, platelets 160×10^9/l The blood film showed anisopoikilocytosis of the red cells with 'tear-drop forms platelets included abnormal giant forms and some megakaryocytic nuclear fragments were seen. Neutrophil alkaline phosphatase score was 8 (normal 35–100) Direct antiglobulin test was negative. Serum vitamin B_{12} was > 1,000 ng/l and folate 6.2 μg/l Sternal marrow on two punctures was difficult to aspirate and hypocellular with normoblastic erythropoiesis and an increased number of primitive reticulum cells. Iliac crest marrow biopsy showed depression of all normal elements, large numbers of fibroblast like cells and an increase in megakaryocytes. Cultures of marrow for chromosome analysis failed but no Ph chromosome was seen in peripheral blood. Chest X-ray was normal, blood urea 6 mmol/l and uric acid 0 18 mmol/l. Other investigations including a range of auto-immune tests were negative. The diagnosis was atypical myelofibrosis with deep venous thrombosis in the left leg, all signs of the latter clearing with high elevation of the limb

Between 1971 and 1976 she remained generally well but with recurrent superficial phlebitis in both legs. The Hb level varied from 8 to 10 g/dl and reticulocytes from 3 to 10%, tending to rise. The leuco-erythroblastic blood picture persisted and the spleen enlarged beyond the umbilicus. In November 1972 the serum folate level was 2.4 μg/l and folic acid 10 mg daily was given. In April 1976 she had symptoms of hypothyroidism, serum thyroxine was under 20 nmol/l and TSH 54 μU/ml thyroxine was given, rising to a maintenance dose of 150 μg daily At this time Hb was 7 g/dl and she had her first transfusion of packed red cells. In August 1976, Hb being 8 g/dl and reticulocytes 8%, prednisolone was started and continued in a dose of 2.5 mg three times daily though the mechanism of any haemolysis was almost certainly hypersplenic: the direct antiglobulin test remained negative or doubtfully positive. However prednisolone appeared to give symptomatic improvement and Hb rose again to 10 g/dl In March 1977 serum urate was raised at 0.51 mmol/l.

In August 1977 she was admitted with left iliofemoral vein thrombosis giving gross oedema which cleared with high elevation, bandaging and 6 days of intravenous heparin. Blood examination showed Hb 6.7 g/dl, 11.6 g after transfusion, WBC 5.0×10^9/l with 10% blast cells, and platelets 75×10^9/l blood urea was 7.6 mmol/l, urate 0.51 mmol/l creatinine 98 μmol/l. On 3 October 1977 she was symptom-free but Hb was 7.8 g dl.

On 11 November 1977 she was readmitted with a history of dribbling urinary incontinence for 4 weeks, and probably oliguria which was obvious after admission, urinary output falling from 300 to less than 100 ml/day The urine contained both protein and blood. There was severe cardiac failure with gross generalised oedema, all of the signs of failure except the oedema improving after Hb was raised from 5 to 8 g/dl by transfusion of packed cells. At this time the WBC was 5.7×10^9/l with 19% myeloblasts neutrophil alkaline phosphatase score was 179 with activity in only 47% of cells, the rest having very low or absent activity which suggested two populations of neutrophils. Platelets numbered 72×10^9/l Sternal marrow gave inconclusive results but chromosome changes were grossly abnormal and had diagnostic implications. Blood urea rose from 36 to 70 mmol/l, urate from 0.95 to 1.3 mmol/l, and creatinine was 814 μmol/l. Total serum protein was 64 g/l and albumin 30 g/l. An intravenous pyelogram gave no excretion and ultrasonic scanning showed bilateral hydronephrosis. She died on 19 November 1977

Autopsy Report

Autopsy examination was limited to abdominal contents. The liver (2,380 g) was enlarged but macroscopically normal. The spleen (870 g) was enlarged and on section was of uniform red appearance. There was bilateral hydronephrosis and hydroureter. The bladder wall was uniformly pale and thickened, in places measuring up to 1.5 cm with granular and haemorrhagic mucosa. There was no lymphadenopathy and no evidence of thrombus in the pelvic veins. The other abdominal viscera were normal.

A sample of vertebral *marrow* showed the typical features of myelofibrosis with extensive fibrosis and only occasional fat spaces (fig. 1). Megakaryocytic hyperplasia was a feature but other marrow elements were very scanty. There was widespread myeloid metaplasia throughout the *liver* and *spleen* and the latter also showed fibrosis of the red pulp. The gross thickening of the *bladder* wall was due to infiltration of the muscularis and mucosa by blast cells although other marrow elements, in particular megakaryocytes, were identifiable (fig. 1b). There was no accompanying fibrosis. Sections from the *kidneys* showed acute pyelonephritis with, in many fields, leukaemic infiltration of both cortex and medulla. A similar infiltrate involved the ovaries, the splenic hilum, and to a lesser extent the myometrium.

Cytogenetic Findings

In 1971 cytogenetic preparation of bone marrow both direct and after culture for 24 h was unsuccessful, possibly owing to an inadequate sample. Blood culture revealed an apparently normal female karyotype in 18 orcein-stained cells.

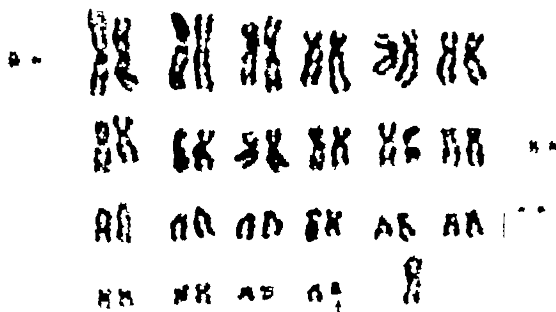
In 1977 bone marrow preparation showed abnormal chromosomes in every cell examined by orcein and by sequential G and C banding. 24 h unstimulated blood cultures gave many metaphases with the same spectrum of abnormalities as found in bone marrow. In 48 and 72 h PHA-stimulated cultures abnormalities were observed but they were less bizarre and normal karyotypes were present in two thirds of these metaphases. Polyploidy was not a feature in either blood or bone marrow.

A Philadelphia-like chromosome (Ph¹) was present (fig. 4a) which apparently took part in satellite association at both telomeres (fig. 2a) suggesting that nucleolus organising sequences were present on both arms, unlike the true Ph¹ chromosome. Furthermore the typical Ph¹ chromosome is usually the result of a reciprocal translocation between chromosomes 22 and 9 [*Watt and Page* 1978] making one chromosome 9 longer. Here the chromosome 9's, identified by sequential G and C banding, were of normal appearance (fig. 4a). Also, some cells had four apparently normal G group chromosomes but one or more D group chromosomes missing, suggesting an alternative origin of the tiny marker.

A large acrocentric marker (fig. 2b) was found in addition to the Ph¹-like element in some cells. It was never seen to take part in satellite association so that its origin from a normal acrocentric is doubtful.

Figure 3 shows partial karyotypes illustrating other markers formed through translocation between chromosomes 1 and 2, one marker mostly composed of the short arm of chromosome 1, another formed from the short arm of a chromosome 2 and the

Fig. 4. G-banded karyotypes from bone marrow with some sequential banding. Showing the Ph¹-like marker and apparently normal 1, 9 and 16 chromosomes confirmed by sequential C-banding. The absence of the X chromosome here was not a consistent abnormality. b Multiple abnormalities and marker chromosomes.



(C b.)

1p 2p 6p

n1 1q 7q

−7 ?10q 18q

? 15q 17q

(C b.)

−21 ? Ph-like chromosome ring

Autopsy Report

Autopsy examination was limited to abdominal contents. The liver (2,390 g) was enlarged but macroscopically normal. The spleen (870 was enlarged and on section was of uniform appearance. There was bilateral hydronephrosis and hydroureter. The bladder w ll w uniformly pal and thickened, in places measuring up to 1.5 cm with granular and haemorrhagic mucosa. There was no lymphadenopathy and no evidence of thrombus in the pelvic eins. The other bdominal viscera were normal.

A sample of vertebral marrow showed the typical features of myelofibrosis with extensive fibrosi and only occasional fat spaces (fig 1) Megakaryocytic hyperplasia was feature but other marrow elements were ery scanty There was widespread myeloid metaplasia throughout the li er and spleen, and the latter also showed fibrosis of the red pulp The gross thickening of the bladder all was due to infiltration of the muscularis and mucosa by blast cells, although other marrow elements, in particular megakaryocytes, were identifiable (fig. 1b) There was no accompanying fibrosis. Sections from the kidney showed acut pyelonephritis with, in many fields, leukaemic infiltration of both cortex and medulla. A similar infiltrate involved the ovaries, the splenic hilum, and to lesser extent the myometrium.

Cytogenetic Findings

In 1971 cytogenetic preparation of bone marrow both direct and after culture for 4 h was unsuccessful, possibly owing to an inadequate sample. Blood culture revealed an apparently normal female karyotype in 18 orcein-stained cells.

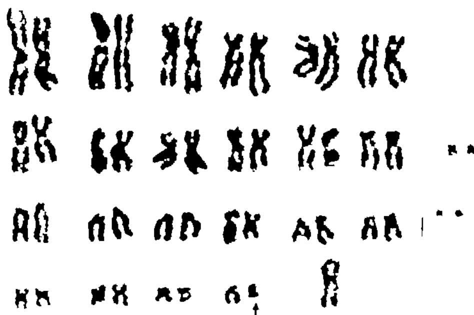
In 1977 bone marrow metaphases howed abnormal chromosomes in every ell examined by orcein and by sequential G and C banding. 24 h unstimulated blood ultures gave many metaphases with the same spectrum of abnormalities as found in bone marrow In 48 and 72 h PHA-stimulated cultures abnormalities were also observed but they were less bizarre and normal karyotypes were present in two thirds of these metaphases. Polyploidy was not a feature in either blood or bone marrow

A Philadelphia like chromosome (Ph) was present (fig. 4a) which apparently took part in satellite association at both telomeres (fig. 2a) suggesting that nucleolus organising sequences were present on both arms, unlike the true Ph chromosome. Furthermore, the typical Ph chromosome is usually the result of a reciprocal translocation between chromosomes 22 and 9 [*Watt and Page* 1978] making one chromosome 9 longer Here the chromosome 9 s, identified by sequential G and C banding, were of normal appearance (fig 4a). Also some cells had four apparently normal G group chromosomes but one or more D group chromosomes missing, suggesting an alternative origin of the tiny marker

A large acrocentric marker (fig. 2b) was found in addition to the Ph -like element in some cells. It was never seen to take part in satellite association so that its origin from a normal acrocentric is doubtful.

Figure 3 shows partial karyotypes illustrating other markers formed through translocation between chromosomes 1 and 2 one marker mostly composed of the short arm of chromosome 1 another formed from the short arm of a chromosome 2 and the

Fig. 4. G-banded karyotypes from bone marrow with some sequential banding. Showing the Ph -like marker and apparently normal 1, 9 and 16 chromosomes confirmed by sequential C-banding. The absence of the X chromosome here was not consistent abnormality b Multiple abnormalities and marker chromosomes.



(C b.)

1p 2p 6p

n1 1q 7q

−7 ?10q 18q

? ? 15q 17q

(C b.)

? −21

Ph−1 like chromosome

ring

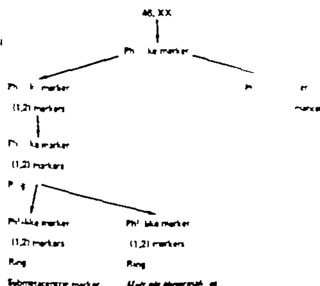


Fig. 5. Possible clonal evolution of some of the marker chromosomes present in the patient.

Ring chromosomes are not often found in acquired chromosome abnormality especially in untreated malignant haematological disorders [*Alimena et al.* 1975] probably because replication is difficult so that rings are at a selective disadvantage. The one present here appeared later in clonal evolution, probably terminally but was well established.

Using the information provided by PHA-stimulated metaphases in peripheral blood, we were able to work out a likely pattern of karyotypic evolution (fig. 5). The reasoning behind this procedure is that blood lymphocytes are long-lived, so that mitotic stimulation yields both pre-morbid lymphocytes and lymphocytes representing chromosome changes that occurred early in the disease process. This explanation is supported by two-thirds of blood metaphases being karyotypically normal and by the finding of a typical Ph chromosome in a proportion of lymphocytes from patients with long-established CML [*Barr and Watt* 1978].

Whether the positive chromosomal findings are helpful in defining a disease so vague as myelofibrosis is not clear but in view of the arbitrary manner of grouping these undifferentiated myeloproliferative disorders [*Laszlo* 1975, *Nowell et al.* 1976] accurate cytogenetic information could well help. The significance of the karyotypic evolution in relation to prognosis may be obscured by treatment including therapeutic irradiation [*Nowell et al.* 1976]. Further the same authors stated that the finding of abnormal clones in myelofibrosis does not mean that leukaemia is imminent, but their series was small and the follow-up period short. Indeed, to achieve such diverse abnormalities in every bone marrow metaphase as well as one-third of PHA-stimulated lymphocytes (where there was a less extreme pattern) our patient must have had chromosome abnormalities for some consid-

long arm of a chromosome 1 an unidentified ring and a submetacentric marker also of unknown origin This figure illustrates how they were combined in different metaphases. For example, the ring was never seen without the (1 2) markers nor the unidentified submetacentric without all three The large acrocentric marker (fig. 2b) was not seen with any of the markers illustrated in figure 3 Some metaphases had more abnormal chromosomes than intact ones. Figure 4b has no more than 36 normal chromosomes out of 48

Discussion

The patient, with otherwise classical features of myelofibrosis, was very unusual not only in the development of extreme chromosome abnormalities, but also in her final clinical presentation with nephropathy and renal failure

In myelofibrosis, aggressive and invasive extramedullary haematopoiesis has been described in a wide variety of organs [*Cehreli et al* 1976 *Glew et al* 1973 *Laszlo* 1975 *Lieberman et al* 1965 *Redlin et al.* 1976 *Ward and Block* 1971] To our knowledge invasion of the bladder wall with myeloblastic transformation of the infiltrate has not been previously described.

Although chromosome abnormality is not typical of myelofibrosis and myelosclerosis, non-specific cytogenetic changes have been reported [*Engel et al* 1968 *Khan and Martin* 1968 *Marsh et al* 1974 *Mitus et al* 1969 *Nowell et al* 1976 *Van Slyck et al* 1970] In many of these the diagnostic categories are not clear and treatment by cytotoxic drugs or radiotherapy may have been contributory In a review *Cehreli et al* [1976] described a case similar to ours in that the marker chromosomes were found in a sample taken in the blastic phase of the disease But, unlike our case, the transformation involved mainly the lymphoid tissues It is also worth noting that in our patient the chromosome abnormalities were found in the absence of cytotoxic drugs or radiation.

Sequential G and C banding is essential in the routine examination of acquired chromosome abnormality [*Page and Watt* 1978] In our patient, for example, the small metacentric marker could easily have been wrongly diagnosed as a Ph[1] chromosome, and similarly the small markers described by *Rudders and Kilcoyne* [1974] and *Khan and Martin* [1968] cannot be called Ph[1] chromosomes without banding. The minute chromosome described by *Cehreli et al* [1976] was cautiously and correctly labelled Philadelphia-like by the authors because no recipient translocation could be resolved by G banding. The value of sequential banding is further demonstrated in that the t (1 2) which produced a (2p 1q) marker in our patient was easily recognised even in poorly preserved cells, but without the banding technique might have been mistaken for a normal chromosome 2. Abnormalities involving chromosome 1 were found in 3 of 8 patients of *Nowell et al* [1976] with chromosome abnormalities from a series of 18 patients with myelofibrosis

When so many chromosomes are abnormal the precise documentation of break points and origins of markers is still remarkably difficult. Where only a few cells contain certain markers the chance of obtaining good staining together with good spreading is more remote than when there is a congenital chromosome abnormality

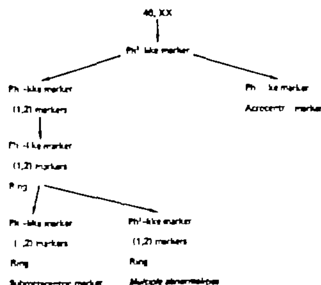


Fig. 5. Possible clonal evolution of some of the marker chromosomes present in the patient.

Ring chromosomes are not often found in acquired chromosome abnormality especially in untreated malignant haematological disorders [*Allmena et al.* 1975] probably because replication is difficult so that rings are at a selective disadvantage. The one present here appeared later in clonal evolution, probably terminally but was well established.

Using the information provided by PHA-stimulated metaphases in peripheral blood, we were able to work out a likely pattern of karyotypic evolution (fig. 5). The reasoning behind this procedure is that blood lymphocytes are long-lived, so that mitotic stimulation yields both pre-morbid lymphocytes and lymphocytes representing chromosome changes that occurred early in the disease process. This explanation is supported by two-thirds of blood metaphases being karyotypically normal and by the finding of a typical Ph chromosome in a proportion of lymphocytes from patients with long-established CML [*Barr and Watt* 1978]

Whether the positive chromosomal findings are helpful in defining a disease so vague as myelofibrosis is not clear but in view of the arbitrary manner of grouping these undifferentiated myeloproliferative disorders [*Laszlo* 1975 *Nowell et al.*, 1976] accurate cytogenetic information could well help. The significance of the karyotypic evolution in relation to prognosis may be obscured by treatment including therapeutic irradiation [*Nowell et al* 1976] Further the same authors stated that the finding of abnormal clones in myelofibrosis does not mean that leukaemia is imminent, but their series was small and the follow-up period short. Indeed, to achieve such diverse abnormalities in every bone marrow metaphase as well as one-third of PHA-stimulated lymphocytes (where there was a less extreme pattern) our patient must have had chromosome abnormalities for some consid-

erable time *Cehreli et al* [1976] implied that results from the study of chromosomes from patients in the blastic phase cannot be compared with chromosome anomalies described in patients with uncomplicated myeloid metaplasia. Few patients with myelofibrosis transform to acute leukaemia [*Glew et al* 1973] but the cytogenetic changes in our patient, supported by the pathological findings suggest that transformation must have occurred. The clonal evolution of karyotype is in keeping with the prominent chromosome instability typical of malignant cells Most tumours appear to evolve by non random losses and/or gains of particular homologues, groups or markers [*Miles* 1974] Indeed in animal experiments the appearance of certain markers has been linked to oncogenic causative agents [*DiPaolo* 1977]

The neutrophil alkaline phosphatase score variable in myelofibrosis, is usually normal or high [*Glew et al* 1973] but low figures more typical of CML [*Damashek and Gunz*, 1964] have been reported in as many as 10–25% of patients in some series [*Mitus et al* 1958 *Ward and Block* 1971 *Laszlo* 1975] However neutrophil function in myeloid metaplasia has not been studied in such detail as in CML [*El Maallem and Fletcher* 1977] It is tempting to speculate that the change from low levels of neutrophil alkaline phosphatase activity to a mixed population of neutrophils with high or very low levels is related to the karyotypic evolution We do not yet know the map position of alkaline phosphatase, although acquired chromosome changes have been used for the purpose of gene mapping Thus, *Marsh et al* [1974] found rhesus-negative erythrocytes in the blood of a rhesus-positive man who had myelofibrosis and related the observation to an acquired 1p+ 4q– translocation, supporting the notion that rhesus is on the short arm of chromosome 1

Acknowledgements

Appreciation is due to Mrs. *Irene Bullock* and Mr *Gordon S Stephen* for excellent technical assistance. *Jessie L. Watt* was supported by SRC Research Studentship B/74/208.

References

Alimena, G Annino, L., Dallapiccola, B., and Ricci, N.. Ring chromosomes and leukaemia. Experientia *31* 1359–1360 (1975).

Barr R. D and Watt, J L.. Preliminary evidence for the common origin of a lympho-myeloid complex in man. Acta haemat. *60* 29–35 (1978)

Cehreli, C. Erdinli, E. Z. Li, C. Y and Kmpotic, E.. Blastic phase of agnogenic myeloid metaplasia simulating malignant lymphoma. Cancer *38* 1297–1305 (1976)

Cohen, S. M.. Chronic myelogenous leukaemia with myelofibrosis. Archs intern. Med. *119* 620–625 (1967)

Damashek, W and Gunz, F.. Leukaemia 2nd ed. (Grune & Stratton, New York 1964).

DiPaolo, J A.. Chromosomal alterations in carcinogen transformed mammalian cells; in The molecular biology of the mammalian genetic apparatus, vol. 2, pp 205–227 (Elsevier/North-Holland, Amsterdam 1977)

El Maallem, H and Fletcher J Impaired neutrophil function and myeloperoxidase deficiency in myeloid metaplasia. Br J Haemat *37* 323–329 (1977)

Engel, W Merker H Schneider G and Wolf, U Clonal occurrence of a chromosome Dq- in myelosclerosis with myeloid metaplasia. Humangenetik *6* 335–337 (1968)

Glew R. H., Haese, W H and McIntyre, P A.. Myeloid metaplasia with myelofibrosis. The clinical spectrum of extramedullary haematopoiesis and tumor formation. Johns Hopkins med. J *132* 253–270 (1973).

Khan, M. H. and Martin, H.: Multiple chromosomal aberrations in a case of malignant myelosclerosis. Acta haemat. 39: 299–308 (1968).
Laszlo, J.: Myeloproliferative disorders (MPD): myelofibrosis, myelosclerosis, extramedullary hematopoiesis, undifferentiated MPD and hemorrhagic thrombocythemia. Semin. Hemat. 12: 409–432 (1975).
Lieberman, P. H.; Rosvoll, R. V., and Ley, A. B.: Extramedullary myeloid tumors in primary myelofibrosis. Cancer 18: 727–736 (1965).
Marsh, W. L.; Chaganti, R. S. K.; Gardner, F. H.; Mayer, K.; Nowell, P. C., and German, J.: Mapping human autosomes: evidence supporting assignment of rhesus to the short arm of chromosome No. 1. Science, N.Y. 183: 966–968 (1974).
Miles, C. P.: Non-random chromosome changes in human cancer. Br. J. Cancer 30: 73–85 (1974).
Mitus, W. J.; Bergna, L. J.; Mednicoff, E. D., and Damashek, W.: Alkaline phosphatase of mature neutrophils in chronic forms of the myeloproliferative syndrome. Am. J. clin. Path. 30: 285–294 (1958).
Mitus, W. J.; Coleman, N., and Kiossoglou, K. A.: Abnormal (marker) chromosomes in two patients with acute myelofibrosis. Archs intern. Med. 123: 192–197 (1969).
Nowell, P.; Jensen, J.; Gardner, F.; Murphy, S.; Chaganti, R. S. K., and German, J.: Chromosome studies in preleukemia. III. Myelofibrosis. Cancer 38: 1873–1881 (1976).
Page, B. M. and Watt, J. L.: The benefits of sequential G and C banding of cancerous metaphases. Abstract. Clin. Genet. (in press, 1978).
Redlin, L.; Francis, R. S., and Orlando, M. M.: Renal abnormalities in agnogenic myeloid metaplasia. Radiology 121: 605–608 (1976).
Rudders, R. A. and Kilcoyne, R. F.: Myeloproliferative disorder with lytic osseous lesions and chromosomal anomalies. Am. J. clin. Path. 61: 673–679 (1974).
Van Slyck, E. J.; Weiss, L., and Dully, M.: Chromosomal evidence for the secondary role of fibroblastic proliferation in acute myelofibrosis. Blood 36: 729–735 (1970).
Ward, H. P. and Block, M. H.: The natural history of agnogenic myeloid metaplasia (AMM) and a critical evaluation of its relationship with the myeloproliferative syndrome. Medicine, Baltimore 50: 357–420 (1971).
Watt, J. L.; Hamilton, P. J., and Page, B. M.: Variation in the Philadelphia chromosome. Hum. Genet. 37: 141–148 (1977).
Watt, J. L. and Page, B. M.: Reciprocal translocation and the Philadelphia chromosome. Hum. Genet. 42: 163–170 (1978).

Received: October 10, 1978
Accepted: November 17, 1978

Jesse L. Watt,
Department of Genetics,
University of Aberdeen, Medical Buildings,
Foresterhill, Aberdeen, AB9 2ZD (Scotland)

Acta haemat. 61 310–316 (1979)

Y Chromosome Duplication A Minor Route Evolutive Pattern in CML

G Alimena, F Mandelli A Montuoro R Gastaldi and B Dallapiccola
Departments of Haematology and Medical Genetics, University of Rome, Rome

Key Words. Blastic transformation Clonal evolution Sex chromosomes Y chromosome

Abstract. 2 patients have been investigated, in which Y chromosome duplication occurred during blastic transformation of chronic myelocytic leukaemia. Comparison of cytogenetic findings and survival data in our cases and in previously reported individuals, suggests preliminary conclusions about the prognostic significance of this aneuploidy Y chromosome gain does not seem to represent *per se* an unfavourable event, unless it is associated with additional chromosome change

Several speculations concerning the biologic and prognostic implications of Y chromosome loss occurring during the evolution of chronic myelocytic leukaemia (CML) have been reported There is a rather general agreement about a longer survival time in male patients missing the Y chromosome as compared with those maintaining the Y chromosome [5 20–22] However a recent review [4] has raised some criticism about these conclusions, and the suggestion that the absence of the Y chromosome protects patients from entering a blastic phase [20 22] has been reconsidered

The Y chromosome duplication seems to occur as a rare evolutive pattern of CML, and no speculation has been so far derived concerning the importance of this aneuploidy

In this paper two additional observations of Y chromosome duplication detected at the onset of blastic crisis (BC) of CML are reported Comparison of these cases with the literature data suggest preliminary conclusions about the prognostic meaning of the Y chromosome gain

Methods

Chromosome preparations were obtained both on short-term peripheral blood cultures in the presence of phytohaemagglutinin (PHA) and by direct technique from bone marrow aspirates, following a 2 h incubation at 37 C in the presence of colchicine. Slides were prepared with the usual air-drying technique the cells were treated with KCl 0.075 *M* hypotonic solution and fixed in Carnoy' solution before squashing. Chromosome characterization was obtained by means of GAG QFQ CBG and G Y banding techniques [1 3].

Case Reports

Case 1 6[illegible]-year-old phenotypically normal male, was diagnosed to be affected by CML in October 1973 because of history of weakness, eight loss, fever conspicuous splenomegaly and hyperleucocytosis (white blood cells 200,000 µl).

Following busulphan treatment, the white blood cell (WBC) fell to 20,000 µl and the physical condition improved I January 1974 the patient developed thrombocytopenia with skin bruising and mucous membrane bleeding, resistant to the treatment ith corticosteroids and platelet concentrates (platelet count 25,000 l) Therapy with hydroxyurea and antifibrinolytics w started. The patient condition remained unchanged until April 1976 at that time blastic phase was diagnosed because of the appearance of highly undifferentiated cells in the peripheral blood (22%) and bone marrow (45%). A therapeutic protocol including hydroxyurea, 6-mercaptopurine (6-MP) nd prednisone improved the patient's condition the blasts percentage fell to less than 20% in the bone marrow but thrombocytopenia was still persisting, the platelet counts ranging between [illegible]3,000 and 50,000 l. The disease remained clinically stable until March 1977 when it deteriorated ith to ic fever heart troubles, increasing WBC to 700,000 l, with 70% blast cells and haemorrhages. The patient died in May 1977. Autopsy permission was not obtained.

Cytogenetic investigations: chromosome analy-

Table I. Chromosomal findings in bone marrow nd peripheral blood preparations in case 1

Date	Tissue	Examined cells	Karyotypes	Cells	
					%
August 1974	bone marrow	30	46, XY	1	3
			46, XY Ph	30	97
	peripheral blood	20	46, XY		90
			46, XY Ph		10
			46, XY	2	9
April 1976	bone marrow	27	47 XYY Ph −7 +21	2	9
			48, XYY Ph +21	23	82
	peripheral blood	25	46, XY	24	96
			48, XYY Ph +21	1	4
June 1976	bone marrow	57	46, XY Ph	2	3 5
			47 XY Ph +21	3	5
			47 XYY Ph	4	7
			48, XYY Ph +21	48	84.5
August 1976	bone marrow	18	47 XY Ph +21	2	12
			48, XYY Ph +21	16	88
October 1976	bone marrow	36	48, XYY Ph +21	36	100
	peripheral blood	15	46, XY	13	87
			48, XYY Ph +21	2	13
January 1977	bone marrow	21	47 XY Ph +21	1	5
			47 XYY Ph −10, +21	1	5
			47 XYY Ph −19 +21	1	5
			48, XYY Ph +21	18	85
March 1977	bone marrow	26	47 XY Ph +21	2	8
			47 XYY Ph −19 +21	1	4
			48, XYY Ph +21	22	84
			48, XYY Ph Ph −19 +21	1	4

Acta haemat. 61 310–316 (1979)

Y Chromosome Duplication A Minor Route Evolutive Pattern in CML

G Allmena F Mandelli A Montuoro R Gastaldi and B Dallapiccola

Departments of Haematology and Medical Genetics, University of Rome, Rome

Key Words. Blastic transformation Clonal evolution Sex chromosomes Y chromosome

Abstract. 2 patients have been investigated in which Y chromosome duplication occurred during blastic transformation of chronic myelocytic leukaemia. Comparison of cytogenetic findings and survival data in our cases and in previously reported individuals, suggests preliminary conclusions about the prognostic significance of this aneuploidy Y chromosome gain does not seem to represent *per se* an unfavourable event, unless it is associated with additional chromosome change.

Several speculations concerning the biologic and prognostic implications of Y chromosome loss occurring during the evolution of chronic myelocytic leukaemia (CML) have been reported. There is a rather general agreement about a longer survival time in male patients missing the Y chromosome, as compared with those maintaining the Y chromosome [5 20–22] However a recent review [4] has raised some criticism about these conclusions and the suggestion that the absence of the Y chromosome protects patients from entering a blastic phase [20 22] has been reconsidered

The Y chromosome duplication seems to occur as a rare evolutive pattern of CML, and no speculation has been so far derived concerning the importance of this aneuploidy

In this paper two additional observations of Y chromosome duplication detected at the onset of blastic crisis (BC) of CML are reported Comparison of these cases with the literature data suggest preliminary conclusions about the prognostic meaning of the Y chromosome gain

Methods

Chromosome preparations were obtained both on short term peripheral blood cultures in the presence of phytohaemagglutinin (PHA) and by direct technique from bone marrow aspirates, following a 2 h incubation at 37 C in the presence of colchicine. Slides were prepared with the usual air-drying technique the cells were treated with KCl 0.075 *M* hypotonic solution and fixed in Carnoy's solution before squashing. Chromosome characterization was obtained by means of GAG QFQ CBG and G-Y banding techniques [1 3]

Table II. Chromosomal findings in bone marrow nd peripheral blood preparations f case 2

Date	Tissue	Examined cells	Karyotypes	Cells	
					%
December 1976	bone marrow	20	46 XY Ph	20	100
			46 XY	2	2
			49 XYY Ph Ph t(6 8)(q34 q11)	34	41
			49 XYY Ph t(6 8)(q34 q11), i(17q)	1	1
			49 XYY Ph Ph t(6 8)(q34 q11), 8	42	50
July 1977	bone marrow	85	49 XYY Ph Ph t(6 8)(q34 q11), (17q)		3
			49 XYY Ph Ph t(6 8)(q34 q11), 19	1	1
			50 XYY Ph Ph t(6 8)(q34 q11), 8, 8, + (17q), +19		2
	peripheral blood	28	46, XY	22	79
			46, XY t(2 15)(q_1 q24)	1	3.5
			47 XYY Ph	4	14
			94 XXYYYY Ph Ph	1	3.5



Fig. 2. G-banded karyotype from case 2, showing Y chromosome duplication and additional chromosome rearrangements (6/8 translocation, isochromosome 17 Ph' duplication)



Fig. 1 G-banded karyotype from case 1 showing a 48, XYY +21 t (9; 22) (q34; q11) chromosome formula (G-bands)

sis of marrow and blood cells were performed on a number of occasions (table I). The first analysis of marrow aspirate during the chronic phase, revealed a 46, XY t (9; 22) (q34; q11) karyotype in 29 out of 30 examined cells. Subsequent data obtained during blastic phase showed an almost homogeneous 48, XYY +21 t (9; 22) (q34; q11) cell population (fig. 1).

In all preparations obtained during blastic transformation, QFQ-banding studies showed three brightly fluorescing regions, staining the distal part of the long arm of Y chromosome and the short arm of a No 14 chromosome, respectively. Y body analysis on bone marrow and peripheral blood smears showed the presence of two fluorescing spots in most cells and three spots in a low percentage of nuclei.

Peripheral blood cultured lymphocytes revealed a 46, XY karyotype with the presence of the heterochromatic 14p+ region, which was segregating in the family. In fact, the same chromosome variant was detected in one of the patient sons. The heterochromatic region was selectively stained by G Y banding [3]. Satellite association studies were not in contrast with the hypothesis that the heavily stained 14p region was a duplicated distal Y long arm.

Case 2 was a 56-year-old phenotypically normal labourer; a patient's brother was affected by trisomy 21 syndrome. He was diagnosed to have CML in December 1976 on the basis of hepatosplenomegaly, anaemia, hyperleucocytosis (WBC 87,000/μl) and the bone marrow morphology. At that time he presented a tumour of the right breast, that histologically appeared to be a leukaemic infiltration by myelocytic precursors.

Following busulphan treatment the condition moderately improved. In June 1977 the disease deteriorated, with appearance of toxic fever, fatigue, diffuse bone pains, liver and spleen enlargement, diffuse lymphadenopathy. The bone marrow aspirate showed blastic transformation with 90% of highly undifferentiated cells. Treatment with hydroxyurea, 6-MP and prednisone was started, but no response to the therapy was achieved and the patient died in August 1977.

Cytogenetic investigations: chromosome analysis of marrow cells were performed on two occasions (table II; fig 2–3). The first preparation, in December 1976, at diagnosis of CML, revealed a 46, XY t (9; 22) (q34; q11) karyotype. The analysis performed in July 1977 at blastic transformation, showed the presence of several clones, the most consistent changes being Y-chromosome and

Table II. Chromosomal findings in bone marrow and peripheral blood preparations in case 2

Date	Tissue	Examined cells	Karyotypes	Cells	
				n	%
December 1976	bone marrow	20	46, XY Ph	20	100
			46, XY	2	2
			48 XYY Ph Ph t(6 8)(q34 q11)	34	41
			48, XYY Ph t(6 8)(q34 q11), i(17q)	1	1
			49 XYY Ph Ph t(6 8)(q34 q11), +8	42	50
July 1977	bone marrow	85	49 XYY Ph Ph t(6 8)(q34 q11), + (17q)	2	3
			49 XYY Ph Ph t(6 8)(q34 q11), +19	1	1
			52, XYY Ph Ph t(6 8)(q34 q11), +8, +8, +i(17q), +19	2	2
	peripheral blood	28	46, XY	22	79
			46, XY t(2 15)(q21 q24)	1	3.5
			47 XYY Ph	4	14
			94 XXYYYY Ph Ph	1	3.5



Fig. 2. G-banded karyotype from case 2, showing Y chromosome duplication and additional chromosome rearrangements (6/8 translocation, isochromosome 17 Ph duplication).

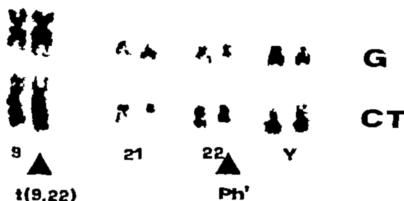


Fig. 3 Partial karyotypes of two cells from case 2, showing Y chromosome duplication and standard 9/22 translocation (G and C T bands).

Table III. Ph -positive cases presenting Y duplication on bone marrow cells

Case	Age years	YY cells, %	Number of chromosomes	Other chromosomal involvement	Diagnosis	Survival months	References
1	27	55	51–57	+ Ph + Cs, + Fs, ± G	BC	3	3
2	59	28	47–51	+ Ph + Cs, + F − G	BC	?	7
3	65	100	47	t(9 13 15 22)	CML	>22	15
4	33	90	53–56	+ Ph + 6, + 10 + 11 + 14 + 19 + 21 + 22	BC		16
5	62	90	47–48	+ Ph + 21	BC	13	personal observation 1
6	56	90	48–49	+ Ph + 8 t(6 8) + i(17q), + 19	BC	3	personal observation 2

Ph duplication and translocation t(6 8) (q25 q13) The analysis of short-term peripheral blood cultures with PHA revealed a normal male karyotype and the presence of sporadic cells with divergent karyotypes.

Discussion

1 case of XYY sex complement developing in bone marrow cells during CML [14] and 3 cases of Y chromosome duplication appearing during blastic crisis [2, 6 15] have been reported previously The attempts to compare the two personal observations with the so far identified cases and to speculate about the prognostic implications of this rare evolutive phenomenon must be cautious because of the patients small sample and the scanty available clinical details.

The main characteristics in the 6 patients are listed in table III Comparison of the cytogenetic findings shows that the presence of a hyperdiploid karyotype is a constant feature In addition to Y chromosome duplication and standard 9/22 translocation, 5 out of 6 cases had Ph duplication in 4 cases C, F or G trisomies occurred and in 2 patients other chromosome translocations were detected

The survival data range between 3 months (cases 1 and 6) and 13 months (case 5) case 3 was still alive in the 22nd month

from diagnosis of CML when we obtained the last information [*Potter* pers. commun 1976]

The prognostic implications of Y chromosome involvement in malignant diseases has been discussed by several authors. Y chromosome loss has often been reported in bone marrow preparations obtained from elderly men [12, 13 21 4] and in patients with CML [4 9 20] BC [10, 11 26] AML [17–20] other haematological disorders [4] and in solid tumours [7 25 28] The exact role of Y chromosome loss is still a matter of discussion and the suggestion that this change is associated with a longer survival in leukaemic patients has been critically reconsidered in recent reports [4 9 11 27] Assuming a more favourable prognosis in cases of Y chromosome loss, an unfavourable clinical evolution could be expected in individuals with Y chromosome duplication. In our 2 patients the survival was 13 and 3 months, respectively from the onset of BC. The first case showed a stable karyotype in bone marrow preparations with trisomy 21 in addition to Y chromosome duplication and standard 9/22 Philadelphia translocation. In the second patient the Y chromosome gain was associated with other chromosome duplications (8 and Ph'), translocations and isochromosome 17 which are *per se* considered to be associated with unfavourable prognostic evolution. In this respect, no conclusions may be derived concerning an hypothetical unfavourable effect of the Y chromosome gain In fact the usual association of Y chromosome aneuploidy with additional karyotypic changes interferes with the understanding of the specific role of these individual aberrations.

Thus, the attempts to subclassify the leukaemias according to their cytogenetic patterns, which have succeeded in a few instances [17–19] are at present inadequate to establish any definite beneficial or unfavourable prognostic effect as far as the Y chromosome duplication is concerned.

References

1 Alimena, G Annino, L., Balestrazzi, P Montuoro, A., and Dallapiccola, B.. Cytogenetic studies in acute leukaemias. Prognostic implications of chromosome imbalances Acta haemat 58 234–239 (1977)

2 Anderson, J W Pearson, G Valdmanis, A., and Mann, J D Duplication of the Y chromosome during myeloblastic crisis of chronic myelocytic leukemia. Annls Génét. *11* 135–137 (1968).

3 Dallapiccola, B and Ricci, N Observations on specific Giemsa staining of the Y and on selective cal destaining of the chromosomes. Humangenetik 26 251 255 (1975).

4 Di Leo, P E Muller H J Obrecht, J P Speck, B., Buhler E M and Stalder G R Loss of the Y chromosome from bone marrow cells of males with myeloproliferative disorders Acta haemat. 57 310–320 (1977).

5 Garson, O M and Milligan, W J The 45, XO Ph subgroup of chronic granulocytic leukemia Scand. J Haematol. 9 186–192 (1972).

6 Goh, K Additional Philadelphia chromosomes in acute blastic crisis of chronic myelocytic leukemia, possible mechanism of producing additional chromosomal abnormalities. Am. J med. Sci. 267 229–240 (1974).

7 Kakati, S., Song, Y S and Sandberg, A. A. Chromosomes and causation of human cancer and leukemia XXII. Karyotypic changes in malignant melanoma. Cancer *40* 1173–1181 (1977).

8 Kiossoglou, K. A., Rosenbaum, E H Mitus, W J and Dameshek, W Multiple chromosomal aberrations in patient with acute granulocytic leukemia associated with Down's syndrome and twinning Blood *24* 134–159 (1964).

9 Lawler S D Lobb, D S and Wiltshaw E. Philadelphia chromosome-positive bone-mar

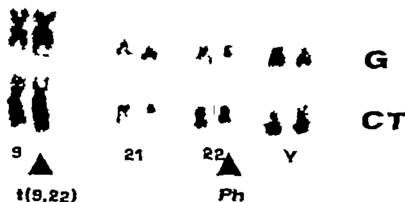


Fig. 3 Partial karyotypes of two cells from case 2, showing Y chromosome duplication and standard 9/22 translocation (G and C T bands).

Table III Ph-positive cases presenting Y duplication on bone marrow cells

Case	Age years	YY cells, %	Number of chromosomes	Other chromosomal involvement	Diagnosis	Survival months	References
1	27	55	51 57	+ Ph + Cs, + Fs, ± G	BC	3	3
2	59	28	47 51	+ Ph + Cs, + F − G	BC	?	7
3	65	100	47	t(9 13 15 22)	CML	> 22	15
4	33	90	53–56	+ Ph + 6 + 10, + 11 + 14 + 19 + 1 + 22	BC	?	16
5	62	90	47–48	+ Ph + 21	BC	13	personal observation 1
6	56	90	48–49	+ Ph + 8 t(6 8) + i(17q), + 19	BC	3	personal observation

Ph duplication and translocation t(6 8) (q25 q13). The analysis of short term peripheral blood cultures with PHA revealed a normal male karyotype and the presence of sporadic cells with divergent karyotypes.

Discussion

1 case of XYY sex complement developing in bone marrow cells during CML [14] and 3 cases of Y chromosome duplication appearing during blastic crisis [2, 6 15] have been reported previously. The attempts to compare the two personal observations with the so far identified cases and to speculate about the prognostic implications of this rare evolutive phenomenon must be cautious because of the patients small sample and the scanty available clinical details.

The main characteristics in the 6 patients are listed in table III. Comparison of the cytogenetic findings shows that the presence of a hyperdiploid karyotype is a constant feature. In addition to Y chromosome duplication and standard 9/22 translocation 5 out of 6 cases had Ph duplication, in 4 cases C, F or G trisomies occurred and in 2 patients other chromosome translocations were detected.

The survival data range between 3 months (cases 1 and 6) and 13 months (case 5); case 3 was still alive in the 22nd month

Acta haemat. 61: 317–324 (1979)

Suppression of Granulopoiesis in Diffusion Chambers by Syngeneic Clonal Acute Myelogenous Leukemia Cells or Peritoneal Exudate Macrophages[1]

Kenneth B. Franklin, William C. Moloney and Joel S. Greenberger

Division of Hematology, Peter Bent Brigham Hospital, Joint Center for Radiation Therapy, Department of Radiation Therapy, Harvard Medical School and Sidney Farber Cancer Institute, Boston, Mass.

Key Words. Acute myelogenous leukemia · Rat leukemia · Marrow suppression

Abstract. To determine the mechanism by which acute myelogenous leukemia (AML) cells suppress normal marrow granulopoiesis, diffusion chambers containing Wistar/Furth (W/Fu) rat marrow cells, peritoneal exudate (PE) macrophages or W/Fu AML clone 3 cells were implanted intraperitoneally into syngeneic irradiated rats. Growth of each population over 21 days in single or double diffusion chambers (in which cell populations were separated by a Nucleopore® filter) was compared to that of mixed populations. Double diffusion chamber culture of homologous or heterologous combinations had no detectable effect on growth kinetics of any of the three cell populations compared to single chambers. In contrast, normal granulocyte proliferation was significantly depressed by single-chamber cocultivation with one tenth the number of PE macrophages or AML cells. Mixing PE macrophages with AML cells produced no preferential population suppression. AML cell differentiation was not detected under any set of conditions. These studies demonstrate that physical contact with proliferating normal macrophages as well as AML cells will suppress granulopoiesis in diffusion chambers.

Introduction

The mechanism of suppression of normal marrow hematopoiesis during leukemogenesis is the subject of intense investigation. Recently diffusible factor(s) derived from some mouse [1] or human [2–5] leukemia cells have been demonstrated to inhibit *in vitro* or diffusion chamber granulopoiesis. However, other diffusion chamber studies have failed to confirm such factor(s) [6] and suggest that direct contact between leukemic and normal cells may be required for suppression of granulocyte proliferation. In the present study, clonally derived Wistar/Furth (W/Fu) rat acute myelogenous

[1] Supported by a grant from the American Cancer Society, Massachusetts Division, Inc.

We thank *Vincent King* and *Mary B. Muse* for excellent technical assistance.

row cells showing loss of the Y in males with chronic myeloid leukaemia. Br J Haemat. *27* 247–252 (1974)

10 Lawler S. D., O Malley F and Lobb, D S. Chromosome banding studies in Philadelphia chromosome positive myeloid leukaemia. Scand J Haematol *17* 17–28 (1976)

11 Nigam, R. and Dosik, H.. Chronic myelogenous leukemia presenting in the blastic phase and its association with a 45 XO Ph karyotype. Blood *47* 223–227 (1976)

12 O'Riordan, M L. Berry E. W and Tough, I M Chromosome studies on bone marrow from a male control population. Br J Haemat. *19* 83–90 (1970)

13 Pierre, R. V and Hoagland, H. C. Age-associated aneuploidy: loss of Y chromosome from human bone marrow cells with ageing. Cancer *30* 889–896 (1972)

14 Potter A. M. Sharp J C. Brown, M J., and Sokol, R. J Structural rearrangement associated with the Ph chromosome in chronic granulocytic leukaemia. Humangenetik *29* 223–228 (1975)

15 Prigogina, E. L. Fleischman, E. W Volkova, M A., and Frenkel M A.. Chromosome abnormalities and clinical and morphologic manifestations of chronic myeloid leukemia. Hum. Genet. *41* 143–156 (1978)

16 Rozynkowa, D Stepien, J., Kowalezki, J and Nowakowski, A. Non-random chromosome rearrangements in 27 cases of human myeloid leukemia. Hum. Genet. *39* 293–301 (1977)

17 Sakurai, M Oshimura, M., Kakati, S., and Sandberg, A. A.. 8-21 translocation and missing sex chromosomes in acute leukaemia. Lancet *ii* 227–228 (1974)

18 Sakurai, M. and Sandberg, A. A.. Chromosomes and causation of human cancer and leukemia. XI Correlation of karyotypes with clinical features of acute myeloblastic leukemia. Cancer *37* 285–299 (1976).

19 Sakurai, M and Sandberg, A. A. Chromosomes and causation of human cancer and leukemia XIII An evaluation of karyotypic findings in erythroleukemia. Cancer *37* 790–804 (1976)

20 Sakurai, M and Sandberg, A. A.. The chromosome and causation of human cancer and leukemia. XVIII. The missing Y in acute myeloblastic leukemia (AML) and Ph-positive chronic myelocytic leukemia (CML). Cancer *38* 762–769 (1976).

21 Sandberg, A A. and Sakurai, M.. The missing Y chromosome and human leukemia. Lancet *i*. 375 (1973)

22 Sandberg, A. A. and Hossfeld, D K.. Chromosomal changes in human tumors and leukemia in Altman, Handbuch der allgemeinen Pathologie, vol. 6 pp 141–287 (Springer Berlin 1974)

23 Schiffman, N J Stecker E. Copen, P E, and Gardner H A.. Males with chronic myeloid leukemia and the 45 XO Ph chromosome pattern. Can. med. Ass. J *110* 1151–1154 (1974)

24 Seeker Walker L. M The chromosomes of bone marrow cells of haematologically normal men and women. Br J Haemat. *21* 455–461 (1971)

25 Selley M. and Vass, L.. Sex chromosome loss in human tumors. Lancet *i* 1041 (1975).

26 Sharp J C. Pötter A. M and Wood, J K.. Non-random and random chromosomal abnormalities in transformated chronic granulocytic leukaemia. Scand. J Haematol. *16*. 5–12 (1976)

27 Wittaker J A. Davies, P and Khurshid, M.. Absence of Y chromosome in patients with chronic granulocytic leukaemia. Acta haemat. *54* 350–357 (1975)

28 Zankl, H. Seidel, H., and Zang, K. D Sex chromosome loss in human tumors. Lancet *i* 221 (1975).

29 Zuelzer W W Thompson, R. I and Mastrangelo, R.. Evidence for a genetic factor related to leukemogenesis and congenital anomalies. chromosomal aberrations in pedigree of an infant with partial D-trisomy and leukemia. J Pediat 72 367–376 (1968)

Received April 3 1978
Accepted October 8, 1978

Dr G Alimena, MD Cattedra di Ematologia, Università di Roma, Via Lancisi 3, I-00161 Roma (Italy)

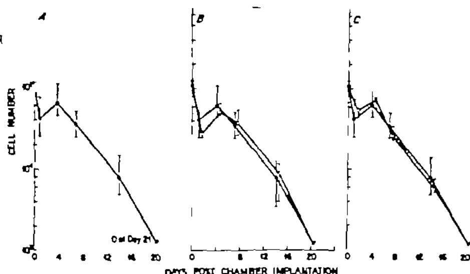


Fig. 1. Effect of double diffusion chamber culture with W/F AML cells or PE macrophages on numbers of PG in normal marrow. The starting cell number (1.5 × 10[illegible]) represents the fraction of the total input (5.0 × 10[illegible]) attributable to PG. A NBM cultured in single chambers. B NBM in double diffusion chambers across from NBM (●) or W/F AML cells (O). C NBM in double diffusion chambers across from NBM (●) or PE macrophages (O).

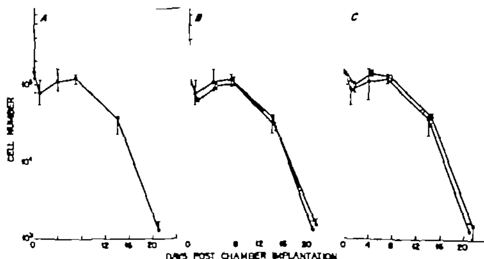


Fig. 2. Effect of double diffusion chamber culture with W/F AML cells or PE macrophages on numbers of NPG in normal marrow. The starting cell number (1.9 × 10[illegible]) represents the fraction of input (5.0 × 10[illegible]) attributable to NPG. A NBM cultured in single chambers. B NBM in double diffusion chamber across from NBM (●) or W/Fu AML cells (O). C NBM in double diffusion chambers from NBM (●) or PE macrophages (O).

leukemia (AML) cells were tested for humoral or contact suppression of syngeneic marrow granulopoiesis. The data indicate that marrow suppression in diffusion chambers by W/Fu AML cells requires cell-cell contact

Materials and Methods

Animals

Female W/Fu rats weighing 120–160 g received 750 rad total-body irradiation, ^{137}Cs source, 663 kV 140 rad/min, 24 h prior to chamber implantation

Diffusion Chambers

Single or double ('piggy-back') diffusion chambers were prepared by the method of *Pfeffer and Boyum* [7] and consisted of lucite rings (13 mm diameter· Millipore, Bedford, Mass.) with Nucleopore® polycarbonate filters (Nucleopore Corp Los Angeles, Calif.) of 0.20 μm porosity Each single chamber or side of a double chamber was filled with 0.1 ml of RPMI 1640 medium containing from 1 0 × 10 to 1.0 × 10⁷ cells. In cell mixing experiments, 0.05 ml containing each of two cell populations was added to total 0 1 ml containing mixed cells. Chamber implantation and harvest were carried out according to published procedures [8, 24] Cells were classified as: *proliferati e granulocytes* (PG) including myeloblasts, promyelocytes, and myelocytes, *no proliferative granulocytes* (NPG) including metamyelocytes, band forms, polymorphonuclear leukocytes and eosinophils *monocyte-macrophages* and *other cells* including reticulum cells, plasma cells, megakaryocytes and mast cells.

AML Cells

A continuous tissue culture line of W/Fu AML clone-3 has been reported [9] Cells were passaged weekly in RPMI 1640 medium supplemented with 10% fetal calf serum (Colorado Serum Co Denver Colo.). Bone marrow cell suspensions were harvested by flushing 150–200 g rat femurs with complete medium Single-cell suspensions were prepared by drawing the marrow through successively smaller gauge needles to a final 30-gauge needle.

Macrophages

For preparation of peritoneal exudate (PE) macrophages 1.0-mm-diameter glass beads w.. autoclaved, washed in sterile saline and 5 g planted intraperitoneally into each of [10] ... gram rats. Cells were harvested 10 days later by washing the peritoneal cavity with ice-cold RPMI-1640 medium and cytocentrifuging the washings. PE cells were then transferred to plastic flasks (Falcon Plastics) and adherent cells removed 12 h later viability tested by trypan blue exclusion, and transferred in 0.05 ml for implantation. This method provided harvests of ≥ 75% macrophage monocytes with ≤ 25% polymorphonuclear leukocytes and eosinophils. After adherence, macrophage populations were ≥ 95% pure.

Histochemistry and Hematologic Staining

Glass coverslip smears were stained with Wright's-Giemsa and tested in cytochemical methods for myeloperoxidase [10], specific 'myeloid' esterase [naphthol-ASD choloroacetate substrate 11], and leukocyte alkaline phosphatase [12 14] according to published procedure [24].

Results

Double-Chamber Culture

The growth of normal rat marrow (NBM) W/Fu AML and PE macrophages in double compared to single chambers was first measured. NBM demonstrated a transient decrease in total cell number at 1 day which increased to input numbers by day 7 and maintained a plateau maximum of approximately 6.5 × 10⁵ cells per chamber to day 21 After day 8 over 50% of cells in the chambers were macrophages with both PG and NPG numbers decreasing (fig. 1a, 2a) Growth in double chambers did not significantly alter these kinetics whether NBM PE macrophages or W/Fu AML cells, occupied the other compartment (table I)

macrophages did not significantly alter ...crophage growth. Thus, nutrient ... through the double chambers was adeq... during the 21-day culture period.

As shown in figure 1a, PG in NBM single chambers decreased over a 21-day period. The kinetics of this decrease were not detectably altered by double-chamber culture with W/Fu AML (fig. 1b) or PE macrophages (fig. 1c). NPG numbers persisted in single NBM chambers for a longer period (fig. 2a) but also decreased rapidly between days 14 and 21. Double-chamber culture ... W/Fu AML cells (fig. 2b) or PE ma...cophages (fig 2c) had no detectable effect ... NPG numbers. NBM cell differential ...unts re...ealed ≥ 50% marrow-derived macrophages by day 8 and ≥ 80% by day ... There was no detectable effect of double-chamber culture with W/Fu AML or PE macrophages on the kinetics of increase in NBM macrophage numbers. Thus, differential as well as total cell numbers in NBM appeared to be unaffected by double-chamber culture with W/Fu AML cells or PE macrophages. Adding a 2-fold higher num-

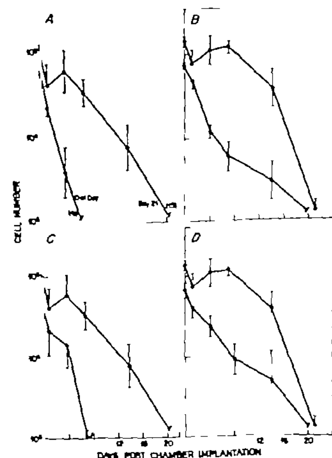


Fig. 3. Effect of mixed chamber culture of 2.5 × 10⁶ cells with 2.5 × 10⁶ W/F AML or PE macrophages. A Numbers of PG in NBM cultured alone (●) or with W/F AML cells (O) B Numbers of NPG in the NBM chambers in A alone (●) or with W/F AML cells (O) C Numbers of PG in NBM cultured alone (●) or with PE macrophages (O). D Numbers of NPG in the NBM chambers in C alone (●) or with PE macrophages (O).

Table I. Suppression of proliferation of NBM granulocytes, by PE macrophages or W/Fu AML Cells in double Diffusion chambers or mixed single chambers

Number of suppressor cells	Marrow granulocytes detected following cocultivation with suppressor cells NBM number on day of harvest $\times 10^{6}$ [a,b]											
	double chamber (separated by filter)						single mixed chamber					
	1	4	8	12	16	21	1	4	8	12	16	20
W/Fu AML clone 3												
1 0 × 10^{6}	5 0	5 1	5 9	1.3	0.3	0.01	0 1	< 0.01	< 0.01	< 0.01	< 0.01	< 0 0
5.0 × 10^{5}	NT						0 1	0 06	< 0 01	NT	NT	NT
2.5 × 10^{5}	5 1	5.9	6.3	1.5	0.4	0 06	0.3	0.2	0.1	0.09	< 0.01	< 0 01
1 0 × 10^{5}	NT						0.8	0 1	0.07	0 03	< 0.01	< 0 0
7.5 × 10^{4}	5 1	5.9	6 7	3 1	0.3	0 02	1 4	1.0	0.6	0 09	< 0.01	< 0 01
5 0 × 10^{4}	NT						2.0	1 1	0.09	0.09	0 03	< 0.01
2.5 × 10	NT						2.5	2.2	0.9	0.6	0.01	< 0.01
1 0 × 10^{1}	NT						2.5	-6	2.1	1 0	0 6	0.5
PE Macrophages												
1.0 × 10^{6}	5 1	6.1	6.3	1 7	0.2	0.03	0.5	< 0.01	< 0.01	< 0 01	< 0.01	< 0 01
5 0 × 10^{5}	NT						1.0	0.5	0.3	0.09	< 0.01	< 0 01
2.5 × 10^{5}	5.3	6.3	5 7	1 7	0.1	0.02	NT					
1 0 × 10^{5}	NT						2.3	1.9	0 9	0 6	0.3	NT
7.5 × 10^{4}	NT						2.5	2.1	2.0	1 0	0.7	NT
5 0 × 10^{4}	5 1	6.3	5.9	1-	0.2	0.01	2.4	3 1	-6	0.5	0.06	NT
2.5 × 10^{4}	NT						2.5	2.4	1.9	0.1	0.1	NT
1.0 × 10^{4}	NT						2.5	3.3	4 0	2.1	0.8	0.2
NBM 1.0 × 10^{6}	5 0	5.2	5.9	1 9	0.09	0.01	NT					
Control, none	5 1	5 6	6.5	2.1	0.1	0.02	2.5	3 1	4 2	1 5	0.09	0.01

NT = Not tested.

Double diffusion chambers, 0.1 ml volume per chamber separated by a Nucleopore filter or single mixed chambers were implanted into 750-rad total-body irradiated rats as described in the Methods. Chambers contained NBM PE macrophages or tissue culture grown W/Fu AML clone 3 cells. Double chambers contained 5 0 × 10^{6} NBM cells and indicated numbers of suppressor cells, single mixed chambers contained 2.5 10^{6} NBM cells and indicated numbers of suppressor cells.

[b] Results are expressed as the mean of NBM cell counts obtained for three chambers. Mixed chamber harvests were scored for the fraction of total cell counts attributable to each population on Wright s-Giemsa-stained differential cell counts of 1,000 cells and corrected to number of NBM granulocytes.

Culture of W/Fu AML clone 3 or PE macrophages in double chambers did not alter the growth of either population compared to its growth in single chambers. W/Fu AML clone 3 cells grew to a plateau of 8 0 × 16 -1 0 × 10^{7} cells/chamber by day 8 while macrophages grew to a plateau more closely resembling NBM (5 0 × 10^{5} cells/chamber) Placing W/Fu AML or NBM cells in double chambers across from

macrophages did not significantly alte rophage growth. Thus, nutrient through the double chambers was adeq during the 21-day culture period.

As shown in figure 1a, PG in NBM s gle chambers decreased over a 21-day per od. The kinetics of this decrease were n detectably altered by double-chamber culture with W/Fu AML (fig. 1b) or PE macrophages (fig. 1c). NPG numbers persisted in single NBM chambers for a longer period (fig. 2a) but also decreased rapidly between days 14 and 21. Double-chamber culture

W Fu AML cells (fig. 2b) or PE ma ges (fig. c) had no detectabl effe NPG numbers. NBM cell differential nt e e ted ≥ 50 marrow-derived rophages by day 8 and ≥ 80 by day Th e was no detectable effect of dou hamber culture with W Fu AML or PE ophages on the kinetics of increase in NBM macrophage numbers. Thus, differential as well as total cell numbers in NBM appeared to be unaffected by double-chamber culture with W/Fu AML cells or PE macrophages. Adding a 2-fold higher num-

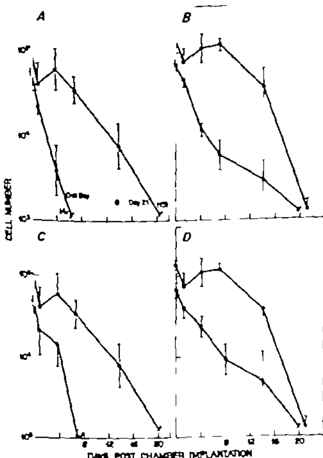


Fig. 3. Effect of mixed chamber culture of 2.5×10^{6} cells with 2.5×10^{6} W/F AML or PE macrophages. A Numbers of PG in NBM cultured alone (●) or with W/F AML cells (O). B Numbers of NPG in the NBM chambers in A alone (●) or with W/Fu AML cells (O). C Numbers of PG in NBM cultured alone (●) or with PE macrophages (O). D Numbers of NPG in the NBM chambers in C alone (●) or with PE macrophages (O).

Table I. Suppression of proliferation of NBM granulocytes, by PE macrophages or W/Fu AML c[illegible] Cells in double Diffusion chambers or mixed single chambers

Number of suppressor cells	Marrow granulocytes detected following cocultivation with suppressor cells NBM number on day of harvest $\times 10^{6}$ [a,b]											
	double chamber (separated by filter)						single mixed chamber					
	1	4	8	1[illegible]	16	21	1	4	8	12	16	20
W/Fu AML clone-3												
1 0 × 10^{6}	5.0	5 1	5.9	1.3	0.3	0 01	0 1	< 0.01	< 0.01	< 0.01	< 0.01	< [illegible]
5 0 × 10^{5}	NT						0 1	0 06	< 0 01	NT	NT	NT
2.5 × 10^{5}	5 1	5.9	6.3	1 5	0.4	0.06	0.3	0.2	0 1	0 09	< 0.01	< 0[illegible]
1 0 × 10^{5}	NT						0 8	0 1	0.07	0.03	< 0.01	< 0 0[illegible]
7.5 × 10^{4}	5 1	5.9	6.7	3 1	0.3	0 02	1 4	1 0	0 6	0.09	< 0 01	< 0 0[illegible]
5 0 × 10^{4}	NT						2.0	1 1	0.09	0 09	0.03	< 0 0[illegible]
[illegible]5 × 10^{4}	NT						2.5	2.2	0.9	0 6	0.01	< 0 01
1 0 × 10^{4}	NT						2.5	[illegible]6	[illegible]1	1 0	0 6	0.5
PE Macrophages												
1 0 × 10^{6}	5 1	6.1	6.3	1 7	0.2	0 03	0.5	< 0 01	< 0.01	< 0.01	< 0.01	< 0 01
5 0 10^{5}	NT						1.0	0.5	0.3	0.09	< 0.01	[illegible] 0 01
2.5 10^{5}	5.3	6.3	5 7	1 7	0 1	0.02	NT					
1.0 × 10^{5}	NT						2.3	1.9	0.9	0.6	0.3	NT
7.5 10^{4}	NT						2.5	2.1	2.0	1 0	0.7	NT
5 0 × 10^{4}	5 1	6.3	5 9	1.2	0[illegible]	0.01	2.4	3 1	2.6	0.5	0.06	NT
2.5 × 10^{4}	NT						[illegible]5	2.4	1.9	0.1	0 1	NT
1 0 × 10^{4}	NT						2.5	3.3	4 0	2.1	0.8	0.2
NBM 1 0 × 10^{6}	5 0	5.2	5.9	1.9	0.09	0 01	NT					
Control none	5 1	5 6	6.5	2.1	0 1	0.02	2.5	3 1	4.2	1.5	0.09	0.01

NT = Not tested.

Double diffusion chambers, 0 1 ml volume per chamber separated by a Nucleopore filter or single mixed chambers were implanted into 750-rad total-body irradiated rats as described in the Methods. Chambers contained NBM PE macrophages or tissue culture grown W/Fu AML clone-3 cells. Double chambers contained 5.0 × 10^{4} NBM cells and indicated numbers of suppressor cells, single mixed chambers contained 2.5 × 10^{5} NBM cells and indicated numbers of suppressor cells.

Results are expressed as the mean of NBM cell counts obtained for three chambers. Mixed chamber harvests were scored for the fraction of total cell counts attributable to each population on Wright s-Giemsa-stained differential cell counts of 1,000 cells and corrected to number of NBM granulocytes.

Culture of W/Fu AML clone 3 or PE macrophages in double chambers did not alter the growth of either population compared to its growth in single chambers. W/Fu AML clone-3 cells grew to a plateau of 8 0 × 16 –1 0 × 10 cells/chamber by day 8 while macrophages grew to a plateau more closely resembling NBM (5 0 × 10 cells/chamber) Placing W/Fu AML or NBM cells in double chambers across from

we in the inhibition of functional assays for committed granulocyte-macrophage progenitor cells (CFUc) or pluripotent stem cells (CFUs, 1) Since functional properties of colony formation in vitro or spleen colony formation are more sensitive to nutritional imbalance, this may explain the positive findings of *Quesenberry et al* [1] Rat CFUc and CFUs assays were not performed in our study and are more difficult to interpret with rat marrow

The W/Fu AML infiltrates rat marrow within 14 days of subcutaneous or 7 days of intraperitoneal inoculation [22] with rapid decrease in hematocrit, peripheral normal white blood cell and platelet counts [3] The present studies indicate that this may be entirely explained by a physiological mechanism which requires cell-cell contact

References

1 Quesenberry P Rappaport, J Fountebuoni, A., Sullivan, R., Zuckerman, K and Ryan, M. Leukemic inhibition of normal murine hematopoiesis. New Engl J Med 299 71 75 (1978).

2 Morris, T C., McNeill, T A and Bridges, J M Inhibition of normal in vitro colony-forming cells by cells from leukemic patients. Br J Cancer 31 641–647 (1975)

3 Chiyoda, S., Mizoguchi, H Asano, S et al Influence of leukaemic cells on the colony formation of human bone marrow cells in vitro. II Suppressive effects of leukaemic cell extracts. Br J Cancer 33 379–384 (1976).

4 Knudtzon, S. and Mortensen, B T Interaction between normal and leukemic human cells in agar culture Scand J Haematol. 17 369–378 (1976)

5 Broxmeyer H. E Jacobsen, N., Kurland, J., Mendelsohn, N and Moore, M A S. In vitro suppression of normal granulocytic cells by inhibiting activity derived from human leukemia cells. J natn. Cancer Inst. 60 497–511 (1978).

6 Miller A M Page P L Hartwell, B. L., and Robinson, S. H. Inhibition of growth of normal murine granulocytes by cocultivated acute leukemia cells. Blood 50 799–809 (1977).

7 Pfeffer P and Boyum A Bone marrow cell culturing in double diffusion chambers. Scand. J Haematol 18 129 136 (1977)

8 Benestad, H B Formation of granulocytes and macrophages in diffusion chamber cultures of mouse blood leukocytes. Scand. J. Haematol 7 279–288 (1970).

9 Greenberger J S Rosenthal, D. S Aaronson, S A and Moloney W C. Acute myelogenous leukemia of the Wistar Furth rat: establishment of continuous tissue culture line producing lysozyme in vitro and in vivo. Blood 46 7 38 (1975).

10 Kaplow S L Simplified myeloperoxidase stain using benzidine dihydrochloride Blood 26 215–221 (1965)

11 Moloney W. L. McPherson, K. and Fliegelman, L. Esterase activity in leukocytes demonstrated by the use of naphthol-AS-D-chloroacetate substrate J Histochem. Cytochem. 8 200 20 (1960)

12 Kaplow S L. A histochemical method for localizing and evaluating alkaline phosphatase activity in disorders of blood and marrow Blood 10 1023–1028 (1955).

13 Menton, M. L. Junge, J and Green, M H. Distribution of alkaline phosphatase in kidney following the use of histochemical azo dye test. Proc. Soc. exp. Biol Med. 57 82–86 (1944)

14 Dusanan, Y Z., Sadowsky E., and Gal, A. Evaluation of the leukocyte alkaline phosphatase activity: comparison of the biochemical and cytochemical method Clinica chim. Acta 34 73–76 (1971)

15 Kurland, J E. and Moore, M. A S. Modulation of hemopoiesis by prostaglandins. Expl Hematol. 5 357–373 (1977).

16 Rothstein, G Hetgl, E H Bishop, C. R., Athens, J W and Ashenbrucker A. L. Stimulation of granulopoiesis by diffusible factor in vivo. J clin. Invest. 50 2004–2007 (1971)

17 Hoelzer D Kurrle, H., Schmucker E. H., and Harriss, E. B.. Evidence for differentiation of human leukemic blood cells in diffusion chamber culture. Blood 49 729–744 (1977).

ber (1.0×10^6) of W/Fu AML or PE macrophages to the other compartment did not alter PG or NPG numbers (table I)

Effect of Mixed-Chamber Culture with W/Fu AML or PE Macrophages on NBM Granulopoiesis

Mixed chambers containing 2.5×10^5 cells of each population were implanted as described in the methods and harvested as above for double chambers. As shown in table I there was a significantly earlier decrease in the numbers of granulocytes in mixed diffusion chambers when an equal number (2.5×10^5) of W/Fu AML cells was present. A decrease was also detected with a 10-fold lower number of W/Fu AML cells (2.5×10^4 table I) Both numbers of PG and NPG cells in mixed chambers decreased rapidly when cocultivated with W/Fu AML cells (fig 3) Mixed cocultivation of NBM with PE macrophages produced granulocyte depression similar to that caused by leukemia cells but less severe (table I) No suppression of NBM was detected if 2.5×10^4 lethally irradiated (2 000 rad, 663 kV 140 rad/min) W/Fu AML or PE macrophages were cocultivated with 2.5×10^5 NBM *cells in single chambers.* W/Fu AML cells have been shown to remain morphologically undifferentiated in diffusion chamber culture [25]

Discussion

The present data demonstrate a decrease in marrow granulocyte growth in diffusion chambers by contact cocultivation with either syngeneic clonal AML cells or normal PE macrophages. While these data are similar to those reported with mouse leukemia cells [6] our observation of significant granulocyte contact suppression by PE macrophages indicates that the phenomenon is not restricted to malignant cells. In contrast to a recent report with mouse leukemia cells [1] no granulocyte suppression was detected if AML cells or macrophages were placed in double chambers separated from the marrow cells by a Nucleopore filter Thus, as in the study by *Miller et al* [6] NBM cell contact with leukemia cells was required.

Release of prostaglandins by macrophages may be the mechanism of suppression of granulopoiesis in diffusion chambers as well as *in vitro* [15] Since the prostaglandin effect can be overcome by colony-stimulating factor [15] and since related diffusable factor(s) flow continuously through the diffusion chamber [5 16] this may explain the absence of detectable macrophage suppression in double diffusion chambers compared to mixed culture An alternate mechanism could be the binding or inactivation of locally active stimulatory substances by the W/Fu AML cells or macrophages. Suppression of granulocytes by contact with PE macrophages may explain granulocyte growth inhibition by marrow macrophages *in diffusion chambers and in vitro* [17–20] Furthermore the only reported *in vitro* system which facilitates long-term granulopoiesis controls macrophage growth by other marrow stromal cells [21] whose balance is maintained by corticosteroids [26]

Marrow suppression in diffusion chambers by W/Fu AML cells required cell-cell contact and occurred prior to W/Fu AML growth plateau. In contrast a humoral inhibitory substance reportedly produced by murine leukemia cells was marrow-suppressive across a double diffusion chamber barrier This substance was most detectably ac

tive in the inhibition of functional assays for committed granulocyte-macrophage progenitor cells (CFUc) or pluripotent stem cells [CFUs 1] Since functional properties of colony formation *in vitro* or spleen colony formation are more sensitive to nutritional imbalance, this may explain the positive findings of *Quesenberry et al.* [1] Rat CFUc and CFUs assays were not performed in our study and are more difficult to interpret with rat marrow

The W/Fu AML infiltrates rat marrow within 14 days of subcutaneous or 7 days of intraperitoneal inoculation [22] with rapid decrease in hematocrit, peripheral normal white blood cell and platelet counts [23] The present studies indicate that this may be entirely explained by a physiological mechanism which requires cell-cell contact.

References

1 Quesenberry P Rappeport, J Fountebuoni, A., Sullivan, R., Zuckerman, K and Ryan, M. Leukemic inhibition of normal murine granulopoiesis. New Engl. J Med. 299 71 75 (1978)

2 Morris, T C., McNeill, T A and Bridges, J M. Inhibition of normal *in vitro* colony-forming cells by cells from leukemic patients. B J Cancer 31 641–647 (1975)

3 Chiyoda, S Mizoguchi, H Asano, S et al Influence of leukaemic cells on the colony formation of human bone marrow cells *in vitro* II. Suppressive effects of leukaemic cell extracts. Br J Cancer 33 379–384 (1976).

4 Knudtzon, S and Mortensen, B T Interaction between normal and leukemic human cells in agar culture Scand. J Haematol. 17 369–378 (1976).

5 Broxmeyer H. E Jacobsen, N., Kurland, J Mendelsohn, N and Moore, M A. S *in vitro* suppression of normal granulocytic cells by inhibiting activity derived from human leukemia cells. J natn. Cancer Inst. 60. 497 511 (1978).

6 Miller A. M., Page, P L., Hartwell, B. L., and Robinson, S. H. Inhibition of growth of normal murine granulocytes by cocultivated acute leukemia cells. Blood 50 799–809 (1977).

7 Pfeffer P and Boyum, A. Bone marrow cell culturing in double diffusion chambers. Scand. J Haematol. 18 129–136 (1977).

8 Benestad, H. B. Formation of granulocytes and macrophages in diffusion chamber cultures of mouse blood leukocytes Scand. J Haematol. 7 279–288 (1970).

9 Greenberger J S Rosenthal, D. S., Aaronson, S. A and Moloney W C. Acute myelogenous leukemia of the Wistar Furth rat, establishment of continuous tissue culture line producing lysozyme *in vitro* and *in vivo* Blood 46. 27–38 (1975).

10 Kaplow S. L. Simplified myeloperoxidase stain using benzidine dihydrochloride Blood 26 215–221 (1965)

11 Moloney W L., McPherson, K., and Fliegelman, L. Esterase activity in leukocytes demonstrated by the use of naphthol-AS-D-chloroacetate substrate. J Histochem. Cytochem. 8 200–210 (1960).

12 Kaplow S. L. A histochemical method for localizing and evaluating alkaline phosphatase activity in diseases of blood and marrow Blood 10 1023–1028 (1955).

13 Menton, M. L., Junge, J and Green, M. H. Distribution of alkaline phosphatase in kidney following the use of histochemical azo dye test Proc. Soc exp Biol. Med. 57 82–96 (1944).

14 Diamant, Y Z Sadovsky E., and Gal, A. Evaluation of the leukocyte alkaline phosphatase activity comparison of the biochemical and cytochemical method. Clinica chim. Acta 34 73–76 (1971).

15 Kurland, J P and Moore, M. A S. Modulation of hemopoiesis by prostaglandins. Expl Hematol. 5 353–373 (1977).

16 Rothstein, G Hetgl, E. H., Bishop, C. R., Athens, J W and Ashenbrucker A. L. Stimulation of granulopoiesis by a diffusible factor *in vivo*. J clin. Invest. 50 2004–2007 (1971).

17 Hoelzer D Kurrle, H., Schmucker E. H., and Harriss, E. B Evidence for differentiation of human leukemic blood cells in diffusion chamber culture. Blood 49 729–744 (1977).

ber (1.0×10^{6}) of W/Fu AML or PE macrophages to the other compartment did not alter PG or NPG numbers (table I).

Effect of Mixed-Chamber Culture with W/Fu AML or PE Macrophages on NBM Granulopoiesis

Mixed chambers containing 2.5×10^{5} cells of each population were implanted as described in the methods and harvested as above for double chambers. As shown in table I, there was a significantly earlier decrease in the numbers of granulocytes in mixed diffusion chambers when an equal number (2.5×10^{5}) of W/Fu AML cells was present. A decrease was also detected with a 10-fold lower number of W/Fu AML cells (2.5×10^{4}, table I). Both numbers of PG and NPG cells in mixed chambers decreased rapidly when cocultivated with W/Fu AML cells (fig. 3). Mixed cocultivation of NBM with PE macrophages produced granulocyte depression similar to that caused by leukemia cells but less severe (table I). No suppression of NBM was detected if 2.5×10^{5} lethally irradiated (2,000 rad, 663 kV, 140 rad/min) W/Fu AML or PE macrophages were cocultivated with 2.5×10^{5} NBM cells in single chambers. W/Fu AML cells have been shown to remain morphologically undifferentiated in diffusion chamber culture [25].

Discussion

The present data demonstrate a decrease in marrow granulocyte growth in diffusion chambers by contact cocultivation with either syngeneic clonal AML cells or normal PE macrophages. While these data are similar to those reported with mouse leukemia cells [6], our observation of significant granulocyte contact suppression by PE macrophages indicates that the phenomenon is not restricted to malignant cells. In contrast to a recent report with mouse leukemia cells [1], no granulocyte suppression was detected if AML cells or macrophages were placed in double chambers separated from the marrow cells by a Nucleopore filter. Thus, as in the study by *Miller et al.* [6], NBM cell contact with leukemia cells was required.

Release of prostaglandins by macrophages may be the mechanism of suppression of granulopoiesis in diffusion chambers as well as *in vitro* [15]. Since the prostaglandin effect can be overcome by colony stimulating factor [15], and since related diffusable factor(s) flow continuously through the diffusion chamber [5, 16], this may explain the absence of detectable macrophage suppression in double diffusion chambers compared to mixed culture. An alternate mechanism could be the binding or inactivation of locally active stimulatory substances by the W/Fu AML cells or macrophages. Suppression of granulocytes by contact with PE macrophages may explain granulocyte growth inhibition by marrow macrophages in diffusion chambers and *in vitro* [17–20]. Furthermore, the only reported *in vitro* system which facilitates long-term granulopoiesis controls macrophage growth by other marrow stromal cells [21] whose balance is maintained by corticosteroids [26].

Marrow suppression in diffusion chambers by W/Fu AML cells required cell-cell contact and occurred prior to W/Fu AML growth plateau. In contrast, a humoral inhibitory substance reportedly produced by murine leukemia cells was marrow-suppressive across a double diffusion chamber barrier. This substance was most detectably ac

the in the inhibition of functional assays for committed granulocyte-macrophage progenitor cells (CFUc) or pluripotent stem cells (CFUs 1) Since functional properties of colony formation *in vitro* or spleen colony formation are more sensitive to nutritional imbalance, this may explain the positive findings of *Quesenberry et al* [1] Rat CFUc and CFUs assays were not performed in our study and are more difficult to interpret with rat marrow

The W/Fu AML infiltrates rat marrow within 14 days of subcutaneous or 7 days of intraperitoneal inoculation [22] with rapid decrease in hematocrit, peripheral normal white blood cell and platelet counts [23] The present studies indicate that this may be entirely explained by a physiological mechanism which requires cell-cell contact.

References

1 Quesenberry P Rappeport, J Fountebuona, A; Sullivan, R., Zuckerman, K., and Ryan, M Leukemic inhibition of normal murine hematopoiesis. New Engl. J Med. 299 71 75 (1978).

2 Morris, T C., McNeill, T A., and Bridges, J M. Inhibition of normal *in vitro* colony-forming cells by cells from leukemic patients Br J Cancer 31 641–647 (1975)

3 Chiyoda, S., Mizoguchi, H., Asano, S et al Influence of leukaemic cells on the colony formation of human bone marrow cells *in vitro* II. Suppressive effects of leukaemic cell extracts Br J Cancer 33 379–384 (1976).

4 Knudtzon, S. and Mortensen, B. T Interaction between normal and leukemic human cells in agar culture. Scand. J Haematol. 17 369–378 (1976).

5 Broxmeyer H. E. Jacobsen, N Kurland, J Mendelsohn, N and Moore, M. A S. *In vitro* suppression of normal granulocytic cells by inhibiting activity derived from human leukemia cells. J natn. Cancer Inst. 60 497–511 (1978).

6 [illegible] A M F [illegible] F [illegible] r ull B L., and F [illegible] n, S H Inhibit[illegible] f growth of nor[illegible] l m[illegible] ne g[illegible] kocytes b[illegible] cultiv ted acute l ker[illegible] 1 Blood 50 7[illegible]–809 (1977)

7 Pfeffer P and Boyum A Bone marrow cell culturing in double diffusion chambers. Scand. J Haema[illegible] 18 129–136 (1977)

8 Benestad, H B Formation of granulocytes and macrophages in diffusion chamber cultures of mouse blood leukocytes Scand. J Haematol 7 279–288 (1970).

9 Greenberger J S Rosenthal, D S Aaronson, S A and Moloney W C Acute myelogenous leukemia of the Wistar Furth rat, establishment of continuous tumor culture line producing lysozyme *in vitro* and *in vivo* Blood 46 27 38 (1975)

10 Kaplow S L Simplified myeloperoxidase stain using benzidine dihydrochloride. Blood 26 215–221 (1965).

11 Moloney W L., McPherson, K and Fliegelman, L Esterase activity in leukocytes demonstrated by the use of naphthol-AS-D-chloroacetate substrate. J Histochem. Cytochem. 8 200–210 (1960).

12 Kaplow S L A histochemical method for localizing and evaluating alkaline phosphatase activity in diseases of blood and marrow Blood 10 1023–1028 (1955).

13 Menten, M. L. Junge, J and Green, M. H. Distribution of alkaline phosphatase in kidney following the use of histochemical azo dye test. Proc. Soc exp. Biol Med. 57 82 96 (1944).

14 Diamant, Y Z Sadovsky E and Gal, A Evaluation of the leukocyt alkaline phosphatase activity comparison of the biochemical and cytochemical method. Clinica chim. Acta 34 73–76 (1971).

15 Kurland, J E. and Moore, M. A. S. Modulation of hemopoiesis by prostaglandins. Expl Hematol. 5 353–373 (1977).

16 Rothstein, G Hergl, E. H., Bishop, C. R., Athens, J W and Ashenbrucker A. L Stimulation of granulopoiesis by diffusible factor *in vivo*. J clin. Invest. 50 2004–2007 (1971).

17 Hoelzer D Kurrle, H Schmucker E. H., and Harriss, E. B., Evidence for differentiation of human leukemic blood cells in diffusion chamber culture. Blood 49 729–744 (1977).

18 Steele, A. A., Sensenbrenner L. L., and Young, M. G.. Growth and differentiation of normal and leukemic human bone marrow cells cultured in diffusion chambers. Expl Hematol. *5* 199–210 (1977).

19 Greenberger J S., Gans, P J King, V., Muse, M B Karpas, A., and Moloney W C. Diffusion chamber culture in irradiated rats of myeloblasts and promyelocytes from 84 untreated patients with myeloid leukemias or refractory dysmyelopoietic anemia. comparison to continuous tissue culture lines, in Bentvelzer Adv comp Leuk. Res. pp 299–302 (Elsevier/North-Holland, Amsterdam 1978).

20 Kurrle, E., Hoelzer P and Harriss, E. B Growth and differentiation of rat bone marrow stem cells in diffusion chambers Expl Hematol. *5* 241–249 (1977).

21 Dexter T M. and Testa, N G.. Differentiation and proliferation of hemopoietic cells in culture· in Methods in Cell Biol., vol 14 pp 387–405 (Academic Press, New York 1976)

22 Greenberger J S. Muse, M. B Bocaccino, C. A., and Moloney W C.. Central nervous system relapse following chemotherapy of W/Fu rat acute myelogenous leukemia. a model for human CNS leukemia. J natn. Cancer Inst. *58* 1139–1146 (1977)

23 Greenberger J S. Lockwood, C. J., France, D S., McGrath, T and Moloney W C.. Total body irradiation and bone marrow transplant in an inbred rat model of acute myelogenous leukemia. Int. J Radiat Oncol. Biol Phys. *4* 421–427 (1978).

24 Greenberger J S., Hassan, L. R. Karpas, A. France, D S., and Moloney W C.. Leukocyte alkaline phosphatase elevation in human acute leukemia derived cell lines cultured in diffusion chambers. Scand. J Haematol. *19* 242–254 (1977).

25 Greenberger J S., Newburger P., Karpas, A., and Moloney W C.. Constitutive and inducible granulocyte-macrophage functions in mouse, rat, and human myeloid leukemia-derived continuous tissue culture lines. Cancer Res. *38* 3340–3348 (1978)

26 Greenberger J S.. Sensitivity of corticosteroid-dependent, insulin-resistant lipogenesis in marrow preadipocytes of mutation diabetic-obese mice. Nature, Lond. *275* 752–754 (1978).

Joel S. Greenberger MD
Joint Center for Radiation Therapy
50 Binney Street
Boston, MA 02115 (USA)

Acta haemat. 61 325–329 (1979)

Present Results of the Cooperative Studies on Aplastic and Refractory Anemias[1]

Y Najean M Le Danvic and A Pecking

Department of Nuclear Medicine, Saint-Louis Hospital, Paris 10e

Key Words. Aplastic anemia Refractory anemia with excess of blasts Androgen therapy prognostic parameters

Aplastic Anemia

Results of the First Cooperative Study

Prognostic Factors

The complementary data gathered in the meantime do not substantially modify the previously published results Individual prognostic factors can be ranked as follows. Infection, granulocyte count, ⁵⁹F utilization, percentage of non-myeloid cells in the bone marrow reticulocyte count and platelet count. A redundancy of these parameters is evident, multiparametric formula has been set up: however when applying it in 88 further cases for whom a 6-month follow-up was available, we observed that 8 of 38 cases classified as mildly severe died within 6 months (79% well classified: of 50 cases classified as highly severe, 24 survived at the 6-month follow-up (52% ell classified): only the extreme values (extremely sever cases and very mild pancytopenia) led to correct prediction, but in such cases any competent physician can establish the correct prognosis at the patient' bed

More interesting is the analysis of the prognostic factors to predict the evolution beyond 3–4 months of therapy Middle and long-term survival cannot be predicted on the basis of initial parameters, nd the multiparametric index does not correlate with survival after 6 months. The evolutive tendency (on androgen therapy in all our cases) was the only useful prognostic factor: even sl ght improvement t 3 months enables one to expect long survival, worsening of even initially mild cases, is of very bad prognostic significance.

Comparison of the Efficiency f Several Androgens

W are now in position to evaluate the results of 70-month period of treatment and to draw some conclusions

The analysis of the results obtained in 57 patients treated with corticosteroids at an approximate dose of 1 mg/kg/day for several months reveals that corticoid treatment does not bring any benefit, either by reducing the hepatic toxicity of androgens or by reducing the frequency or severity of hemorrhages: on the contrary the actuarial survival curves from patients with similar initial severity of disease demonstrat higher mortality in the corticosteroid-treated cases

The survival curves demonstrate statistical differences, according to the drug used ($p<0.1$). The probality of an excess of deaths in the oxymetholone-treated group (2.5 mg/kg/day) during the first 3 months is lower than 0.1 In the patients

[1] This study was published on behalf of the Study Group and will be discussed at the 4th French Congress of Haematology

18 Steele, A. A. Sensenbrenner L. L., and Young, M G Growth and differentiation of normal and leukemic human bone marrow cells cultured in diffusion chambers. Expl Hemat. *5* 199–210 (1977)
19 Greenberger J S. Gans, P J King, V Muse, M. B., Karpas, A., and Moloney W C. Diffusion chamber culture in irradiated rats of myeloblasts and promyelocytes from 84 untreated patients with myeloid leukemias or refractory dysmyelopoietic anemia. comparison to continuous tissue culture lines, in Bentvelzer Adv comp Leuk. Res. pp 299–302 (Elsevier/North-Holland, Amsterdam 1978).
20 Kurrle, E., Hoelzer P and Harriss, E. B Growth and differentiation of rat bone marrow stem cells in diffusion chambers. Expl Hemat. *5* 241–249 (1977)
21 Dexter T M. and Testa, N G. Differentiation and proliferation of hemopoietic cells in culture In Methods in Cell Biol vol. 14 pp 387–405 (Academic Press, New York 1976)
22 Greenberger J S. Muse, M B Bocaccino C. A., and Moloney W C. Central nervous system relapse following chemotherapy of W/Fu rat acute myelogenous leukemia. a model for human CNS leukemia. J natn. Cancer Inst. *58* 1139–1146 (1977).
23 Greenberger J S. Lockwood, C. J., France, D S. McGrath, T and Moloney W C. Total body irradiation and bone marrow transplant in an inbred rat model of acute myelogenous leukemia. Int. J Radiat. Oncol. Biol. Phys. *4* 421–427 (1978)
24 Greenberger J S., Hassan, L. R. Karpas, A., France, D S., and Moloney W C. Leukocyte alkaline phosphatase elevation in human acute leukemia derived cell lines cultured in diffusion chambers. Scand. J Haematol. *19* 242–254 (1977).
25 Greenberger J S., Newburger P Karpas, A., and Moloney W C. Constitutive and inducible granulocyte-macrophage functions in mouse, rat, and human myeloid leukemia-derived continuous tissue culture lines. Cancer Res. *38* 3340–3348 (1978)
26 Greenberger J S. Sensitivity of corticosteroid-dependent, insulin-resistant lipogenesis in marrow preadipocytes of mutation diabetic-obese mice. Nature, Lond. *275* 752–754 (1978)

Joel S Greenberger MD
Joint Center for Radiation Therapy
50 Binney Street
Boston, MA 02115 (USA)

Acta haemat. 61 325–329 (1979)

Present Results of the Cooperative Studies on Aplastic and Refractory Anemias[1]

Y. Najean, M. Le Danvic and A. Pecking

Department of Nuclear Medicine, Saint Louis Hospital, Paris 10e

Key Words. Aplastic anemia · Refractory anemia with excess of blasts · Androgen therapy · prognostic parameters

Aplastic Anemia

Results of the First Cooperative Study

Prognostic Factors

The complementary data gathered in the meantime do not substantially modify the previously published results. Individual prognostic factors can be ranked as follows: infection, granulocyte count, ^{59}Fe utilization, percentage of nonmyeloid cells in the bone marrow, reticulocyte count and platelet count. As redundancy of these parameters is evident, multiparametric formula has been set up; however when applying it in 88 further cases for whom 6-month follow-up was available, we observed that 8 of 38 cases classified as mildly severe died within 6 months (79% well classified); of 50 cases classified as highly severe, 24 survived at the 6-month follow-up (52% well classified): only the extreme values (extremely severe cases and very mild pancytopenia) led to correct prediction, but in such cases any competent physician can establish the correct prognosis at the patient's bed.

More interesting is the analysis of the prognostic factors to predict the evolution beyond 3–4 months of therapy. Middle and long-term survival cannot be predicted on the basis of initial parameters, and the multiparametric index does not correlate with survival after 6 months. The evolutive tendency (on androgen therapy in all our cases) was the only useful prognostic factor: even slight improvement at 3 months enables one to expect long survival, worsening of even initially mild cases, is of very bad prognostic significance.

Comparison of the Efficiency of Several Androgens

We are now in a position to evaluate the results of a 20-month period of treatment and to draw some conclusions.

The analysis of the results obtained in 57 patients treated with corticosteroids at an approximate dose of 1 mg/kg/day for several months reveal that corticoid treatment does not bring any benefit, either by reducing the hepatic toxicity of androgens or by reducing the frequency or severity of hemorrhages: on the contrary, the actuarial survival curves from patients with similar initial severity of disease demonstrate a higher mortality in the corticosteroid treated cases.

The survival curves demonstrate a statistical difference, according to the drug used ($p<0.1$). The probabilty of an excess of deaths in the oxymetholone-treated group (2.5 mg/kg/day) during the first 3 months is lower than 0.1. I the patients

[1] This study was published on behalf of the Study Group and will be discussed at the 4th French Congress of Haematology.

18 Steele, A. A. Sensenbrenner L. L., and Young, M. G. Growth and differentiation of normal and leukemic human bone marrow cells cultured in diffusion chambers. Expl Hemat. 5 199–210 (1977).

19 Greenberger J. S. Gans, P. J. King, V. Muse, M. B. Karpas, A., and Moloney W. C. Diffusion chamber culture in irradiated rats of myeloblasts and promyelocytes from 84 untreated patients with myeloid leukemias or refractory dysmyelopoietic anemia. comparison to continuous tissue culture lines, in Bentvelzer Adv comp Leuk. Res. pp 299–302 (Elsevier/North-Holland, Amsterdam 1978)

20 Kurrle, E. Hoelzer P. and Harriss, E. B. Growth and differentiation of rat bone marrow stem cells in diffusion chambers. Expl Hemat. 5 241–249 (1977).

21 Dexter T. M. and Testa, N. G. Differentiation and proliferation of hemopoietic cells in culture in Methods in Cell Biol vol. 14 pp 387–405 (Academic Press, New York 1976).

22 Greenberger J. S. Muse, M. B., Bocaccino, C. A., and Moloney W. C. Central nervous system relapse following chemotherapy of W/Fu rat acute myelogenous leukemia a model for human CNS leukemia. J natn. Cancer Inst. 58 1139–1146 (1977).

23 Greenberger J. S., Lockwood, C. J. France, D. S. McGrath, T., and Moloney W. C. Total body irradiation and bone marrow transplant in an inbred rat model of acute myelogenous leukemia. Int. J. Radiat. Oncol. Biol. Phys. 4 421–427 (1978)

24 Greenberger J. S., Hassan, L. R. Karpas, A. France, D. S., and Moloney W. C. Leukocyte alkaline phosphatase elevation in human acute leukemia derived cell lines cultured in diffusion chambers. Scand. J. Haematol. 19 242–254 (1977).

25 Greenberger J. S., Newburger P., Karpas, A., and Moloney W. C. Constitutive and inducible granulocyte-macrophage functions in mouse, rat, and human myeloid leukemia-derived continuous tissue culture lines. Cancer Res. 38 3340–3348 (1978)

26 Greenberger J. S. Sensitivity of corticosteroid dependent, insulin-resistant lipogenesis in marrow preadipocytes of mutation diabetic-obese mice. Nature, Lond. 275 752–754 (1978)

Joel S. Greenberger MD
Joint Center for Radiation Therapy
50 Binney Street
Boston, MA 02115 (USA)

Acta haemat. 61 325–329 (1979)

Present Results of the Cooperative Studies on Aplastic and Refractory Anemias[1]

Y. Najean, M. Le Danvic and A. Pecking

Department of Nuclear Medicine, Saint Louis Hospital, Paris 10e

Key Words. Aplastic anemia · Refractory anemia with excess of blasts · Androgen therapy · prognostic parameters

Aplastic Anemia

Results of the First Cooperative Study

Prognostic Factors

The complementary data gathered in the meantime do not substantially modify the previously published results. Individual prognostic factors can be ranked as follows: infection, granulocyte count, 59F utilization, percentage of non-myeloid cells in the bone marrow, reticulocyte count and platelet count. As redundancy of these parameters is evident, multiparametric formula has been set up; however when applying it to 88 further cases for whom 6-month follow-up was available, we observed that 8 of 38 cases classified as mildly severe died within 6 months (79% well classified; of 50 cases classified as highly severe, 24 survived at the 6-month follow-up (52% well classified): only the extreme values (extremely severe cases and very mild pancytopenia) led to correct prediction, but in such cases any competent physician can establish the correct prognosis at the patient's bed.

More interesting is the analysis of the prognostic factors to predict the evolution beyond 3–4 months of therapy. Middle and long-term survival cannot be predicted on the basis of initial parameters, and the multiparametric index does not correlate with survival after 6 months. The evolutive tendency (on androgen therapy in all our cases) was the only useful prognostic factor: even slight improvement at 3 months enables one to expect long survival; worsening of even initially mild cases, is of very bad prognostic significance.

Comparison of the Efficiency of Several Androgens

We are now in a position to evaluate the results of a 20-month period of treatment and to draw some conclusions.

The analysis of the results obtained in 57 patients treated with corticosteroids at an approximate dose of 1 mg/kg.day for several months reveals that corticoid treatment does not bring any benefit, either by reducing the hepatic toxicity of androgens or by reducing the frequency or severity of hemorrhages; on the contrary, the actuarial survival curves from patients with similar initial severity of disease demonstrate a higher mortality in the corticosteroid-treated cases.

The survival curves demonstrate a statistical difference, according to the drug used ($p<0.1$). The probality of an excess of deaths in the oxymetholone-treated group (2.5 mg/kg/day) during the first 3 months is lower than 0.1. In the patients

[1] This study was published on behalf of the Study Group and will be discussed at the 4th French Congress of Haematology.

surviving the 3rd month, further survival of metenolone treated (2.5 mg/kg/day) patients is statistically ($p<0.05$) lower than that of norethandrolone- or methandrostenolone treated (1 mg/kg/day) cases.

The analysis of possible improvement also demonstrates that at 3 6 and 10 months the patients treated with the latter two drugs demonstrate a better rate of improvement than the other cases, this is in agreement with the survival curves.

It is of interest that we did not observe any difference in toxicity whatever the drug used, alkylated or not at C 17

Factors Which Influence the Improvement of Patients on Androgen Therapy

The most interesting factor appears to be the duration of androgen therapy During the third quarter of the first year and even after 12 months, improvement of the red cell line continued in cases with persisting anemia (26 of the 52 cases with Hb lower than 10 g/dl at 6 months were improved at 10 months 9 of the 17 cases with Hb lower than 10 g/dl at 10 months and evaluated at 20 months were improved). The same finding was observed for the granulocyte line (9 of 27 patients with less than 1 000 PNM per microliter at 6 months and still alive at 10 months were improved 8 of the 14 patients with less than 1 000 PNM per microliter at 10 months and evaluated at 20 months were improved as well) Platelet improvement was less evident, but the same late improvement could be noted (16 of the 41 cases with platelet counts lower than ?0,000/µl were improved between 6 and 10 months, and 21 of the 32 thrombocytopenic patients between 10 and 20 months).

From our data it appears that. improvement can start earlier than is often assumed (as soon as the 3rd month, and even at the 6th week) late improvement is possible, particularly on the platelet line, justifying lengthening of the androgen therapy as previously observed, platelet improvement is less frequent and, if it occurs, less complete than granulocyte and red cell improvement.

Long-Term Evol tion

At the present time, only 76 patients, alive at 20 months, and followed for 2 years or more, can be evaluated. Our data cannot yet be published and the present results have to be considered as prel minary

According to the protocol, two ways were used to decrease androgen therapy a fast one (one third of the total dose every month) and a slow one (one tenth every other month), the choice being random. The retrospective analysis of the charts demonstrates that the protocol was not always perfectly observed, but it is clear that the patients allocated to the fast-decrease group relapsed in a larger proportion and earlier than the patients allocated to the slow-decrease group (at 12 months 13 of 31 patients vs. 4 of 27 at 18 months 15 of 30 patients vs. 5 of 25 at ?4 months 16 of 28 patients vs. 8 of 22)

The initial severity does not preclude the chance of a long remission (or of a definite cure) but, as expected, the degree of improvement at the end of the initial high-dose androgen treatment was correlated with the further course of the disease of the 11 deaths observed after 20 months, 8 had been observed in not or incompletely improved patients, and in only 2 had red cell and granulocytic lines normalized (the 11th death was accidental)

Resumption of androgens in case of relapse demonstrates androgen efficacy and dependence, since in 15 or 17 cases evaluated an objective response was observed.

Although our data are not yet complete, our series appears large enough to demonstrate the efficiency of androgens upon the following grounds: the difference in survival and degree of improvement according to the drug used the high rate of relapses (at least 50%) after suspension of the treatment, and the efficacy of androgen resumption in case of relapse.

Preliminary Results of the St dy in Progress

The protocol of this study was established before all the results of the preceding study were available its purpose was to compare high and low-dose androgens since the high doses generally used are not clearly justified and since a recent Japanese study suggested equal efficiency of high and low doses of androgens. A randomization between high dose (1 mg/kg/day methandrostenolone 1 mg/kg/day stanozolol) and low dose (methandrostenolone 0.2 mg/kg/day fluoxymesterone 0.2 mg/kg/day) was chosen. Its purpose was also to use the recent possibilities of bone marrow grafting or immunosuppression by antithymocytic serum in cases in whom no clear improvement

was derived from androgen therapy after reasonable period of time and in whom technical possibilities (age, donor acceptance of the therapy) could permit such tentative.

At the present time only 44 charts can be evaluated (three charts have been excluded because of diagnostic errors: 2 preleukemias, 1 fatal aplastic anemia due to busulfan therapy in polycythemia vera) 38 cases could be classified as severe or very severe, according to the criteria previously used. As it is unlikely that the clinical picture of the disease changed from one year to the other it is probable that the physicians used androgens in the mild cases without randomizing them, and informed the group only of the most severe cases, in whom the administration of antithymocytic serum or bone marrow grafting could have been considered.

In this small series, when excluding the grafted patients and the cases treated with antithymocytic serum, we note that *all the deaths occurred during the first 3 months*, which confirms previous data concerning this critical phase of the disease and the need for adequate therapy during this period.

The decision of using antithymocytic serum or bone marrow grafting was taken in 10 of 15 cases before 4 weeks of androgen therapy before any objective assessment of its efficacy was possible. This particular point needs discussion since it is difficult in severely ill patients, to choose between the risks of waiting for the results of androgen and symptomatic treatment (risk of death from infection or hemorrhage, increased risk of graft rejection in multitransfused patients), and the risk inherent in bone marrow grafting in patients still apt to improve. In that series, antithymocytic serum was inefficient in 5 of 6 cases. Of 13 bone-marrow-grafted cases 9 died.

When analyzing the nongrafted patients, one can notice that 6 of 13 cases *treated with high doses* of androgens and 4 of 14 cases treated with low doses have been alive for 6 months or more; all the surviving patients treated with high doses were improved, but none of the surviving cases treated with low doses. Our present results are certainly too scanty to allow definite conclusions but we think that by extension of the present study the need for sufficiently high dose and sufficiently long period of treatment could possibly be demonstrated.

Refractory Anemia with an Excess of Bone Marrow Blasts

Results of the First Cooperative Study

In the protocol of this study three therapeutic regimens were considered: symptomatic treatment alone, metenolone (2.5 mg/kg day for 10 months), or cytosine-arabinoside (4 consecutive subcutaneous injections at 12-hour intervals, 20 mg/m² each, monthly for 10 months). The patients were assigned to each group at random, whatever the expected evolution.

59 patients could be evaluated, all except 1 aged of more than 45 years.

From this series, it is possible to set forth some prognostic factors: bone marrow blastosis (median survival in the group with less than 20%/ blasts = 14 months; in the group with 20–40% blasts = 8 months; correlation coefficient between survival and initial number of blasts = 0.29): degree of granulocytopenia (correlation coefficient = 0.31: median survival when the granulocyte count was lower than 1,000/μl = 8 months, when it was higher than 1,000/μl = 14 months); platelet count (similar survival in the groups with less than 20,000/μl, comprised between 20,000 and 40,000 and between 40,000 and 80,000; but long survival median 19 months of the cases with an initial platelet count of more than 80,000/μl); iron kinetics (only 1 of the 11 cases with ⁵⁹F incorporation higher than 50%/ died before 15 months, contrasting to 60% of the 12 cases with an incorporation comprised between 30–50%, and 70% of the 20 cases with an incorporation lower than 30%).

However at 20 months, 20%/ of the patients only survived, whatever their initial number of blasts. The presence of few circulating myeloblasts is not definitely correlated with the prognosis: 11 patients died from overt leukemia, but 4 are still alive 12–19 months later. The degree of granulocytopenia and the degree of bone marrow blastosis seem to be of little prognostic value at that time. Only the platelet count and the results of the ⁵⁹F kinetic studies appear to be prognostic factors independent of the percentage of blasts. In this series we have only the results of 13 *in vitro* cultures of bone marrow stem cells and 6 karyotypic studies, so that we cannot test the independence of these factors, as far as their prognostic value is concerned.

surviving the 3rd month further survival of metenolone treated (2.5 mg/kg/day) patients is statistically ($p<0.05$) lower than that of norethandrolone or methandrostenolone-treated (1 mg/kg/day) cases.

The analysis of possible improvement also demonstrates that at 3 6 and 10 months the patients treated with the latter two drugs demonstrate a better rate of improvement than the other cases this is in agreement with the survival curves.

It is of interest that we did not observe any difference in toxicity whatever the drug used, alkylated or not at C 17

Factors Which Influence the Improvement of Patients on And ogen Therapy

The most interesting factor appears to be the duration of androgen therapy During the third quarter of the first year and even after 12 months, improvement of the red cell line continued in cases with persisting anemia (26 of the 52 cases with Hb lower than 10 g/dl at 6 months were improved at 10 months 9 of the 17 cases with Hb lower than 10 g/dl at 10 months and evaluated at 20 months were improved). The same finding was observed for the granulocyte line (9 of 27 patients with less than 1,000 PNM per microliter at 6 months and still alive at 10 months were improved 8 of the 14 patients with less than 1,000 PNM per microliter at 10 months and evaluated at 20 months were improved as well) Platelet improvement was less evident, but the same late improvement could be noted (16 of the 41 cases with platelet counts lower than 20,000/μl were improved between 6 and 10 months, and 21 of the 32 thrombocytopenic patients between 10 and 20 months)

From our data it appears that. Improvement can start earlier than is often assumed (as soon as the 3rd month, and even at the 6th week) late improvement is possible, particularly on the platelet line, justifying lengthening of the androgen therapy· as previously observed, platelet improvement is less frequent and, if it occurs, less complete than granulocyte and red cell improvement.

Long-Term Evolutio

At the present time, only 76 patients, alive at 20 months, and followed for 2 years or more, can be evaluated Our data cannot yet be published and the present results have to be considered as preliminary

According to the protocol, two ways were used to decrease androgen therapy· a fast one (one third of the total dose every month) and a slow one (one tenth every other month), the choice being random. The retrospective analysis of the charts demonstrates that the protocol was not always perfectly observed, but it is clear that the patients allocated to the fast-decrease group relapsed in a larger proportion and earlier than the patients allocated to the slow-decrease group (at 12 months 13 of 31 patients vs. 4 of 27 at 18 months 15 of 30 patients vs. 5 of 25 at 24 months 16 of 28 patients vs. 8 of 22)

The initial severity does not preclude the chance of a long remission (or of a definite cure) but, as expected, the degree of improvement at the end of the initial high-dose androgen treatment was correlated with the further course of the disease· of the 11 deaths observed after 20 months, 8 had been observed in not or incompletely improved patients, and in only 2 had red cell and granulocytic lines normalized (the 11th death was accidental)

Resumption of androgens in case of relapse demonstrates androgen efficacy and dependence, since in 15 or 17 cases evaluated an objective response was observed.

Although our data are not yet complete, our series appears large enough to demonstrate the efficiency of androgens upon the following grounds: the difference in survival and degree of improvement according to the drug used, the high rate of relapses (at least 50%) after suspension of the treatment, and the efficacy of androgen resumption in case of relapse

Prelimin ry Results of th St dy in Progress

The protocol of this study was established before all the results of the preceding study were available· its purpose was to compare high- and low-dose androgens since the high doses generally used are not clearly justif ed and since a recent Japanese study suggested equal efficiency of high and low doses of androgens. A randomization between high dose (1 mg/kg/day methandrostenolone· 1 mg/kg/day stanozolol) and low dose (methandrostenolone 0 2 mg/kg/day fluo ymester one 0.2 mg/kg/day) was chosen. Its purpose wa also to use the recent possibilities of bone marrow grafting or immunosuppression by antithymocytic serum in cases in whom no clear improvement

1 cancers, 1 infection). According to the durat on of the follow-up and the initial parameters (se verity there is no difference between the groups. It becomes mor and mor clea that drugs do not improve refractory anemia with excess of bone marrow blasts. In the present se ries, anemia improved in only 1 patient.

Only 6 patients were treated with the suggested chemotherapy: 5 died, but retrospective analysis demonstrates that in these 5 patients, death oc curred either early in the evolution or chemotherapy was not correctly used: only 1 case received the scheduled monthly courses during the 12 months of treatment and remains alive, without any improvement, however (number of blasts, leucytopenia, need for transfusions, CFC number).

[illegible]
number [illegible]
severity insufficient [illegible]
is high [illegible]
[illegible] than 60% of the patient [illegible] acute leukemia. It then appears ethical to consider the [illegible] tematic use of chemotherapy in all the cases. A new protocol, founded on these data, could be discussed.

Dr. Y. Najean, Departement de médecine nucléaire, Hôpital St Louis, F-75010 Paris (France)

The second interesting information concerns the evolution in the three therapeutic weapons. the actuarial survival curves are absolutely similar in the three groups, which indicates that neither androgen therapy nor cytosine-arabinoside at the dosage used do modify the prognosis. On the other hand, the cause of death was similar in each group (7 deaths from leukemia in the androgen-treated group 9 in the nontreated group 7 in the cytosine-arabinoside-treated group), which indicates that the chemotherapy used is inadequate (or insufficient) and that androgen therapy does not accelerate blast proliferation as it has been suggested

The detailed results will be published elsewhere.

Preliminary Results of the Study in Progress

The protocol of this study in its final version (July 1977) integrates the previous data and suggests offering different therapeutic ways according to the expected severity of the disease, – completing our information on the possible efficacy of androgen therapy and trying to define the possible clinical interest of specific antileukemic treatment.

From the initial evaluation and a 3-month survey without specific therapy it was expected that it could be possible to discriminate between two groups of patients: group A with medullary blastosis constantly below 20%, without blood invasion by blasts and without clinical symptoms of progress, and group B with an increasing percentage of blasts, appearance of circulating blastosis or clinical symptoms of progress. Group A patients had to be followed, according to randomization, on androgen therapy or symptomatic treatment only (with the possibility of joining group B at any time during the survey) group B patients were treated by monthly chemotherapy regimens: vincristine (1.5 mg/m²) on the 1st day hydroxyurea (0.6 mg/m²) every 12 h for 5 days.

At the present time (January 1979), complete data on 30 cases only are available with follow-up of 6 months or more. The following preliminary conclusions can be drawn.

Validity of an a priori *Classification of the Patients*

Only 6 patients were assigned to group B (severe prognosis, chemotherapy), 24 to class A When revising the charts, it was clear that a large fraction of the latter cases exhibited objective class B characteristics (13 of the 24 cases). It is difficult to understand why so many severe cases were considered by their physician as relatively mild. one explanation could be the fear of using 'aggressive chemotherapy including monthly hospitalization and compelling supervision on the other hand, it is generally assumed (without objective proof) that chemotherapy in this anemia is useless and often noxious in old patients (9 of these 13 patients were more than 70 years old)

It is certainly difficult to define clear prognostic criteria. Our data, however suggest relatively clear parameters initially high (more than 20%) or an increasing percentage of blasts in the *bone marrow eventually the presence of blasts in* the circulation, and the degree of granulocytopenia have been previously clearly defined as unfavorable findings, other objective parameters, as cell kinetics (bone marrow autoradiography and ^{59}Fe kinetics), quantification of bone marrow stem cells and karyotypic studies, are used only in a limited number of cases; empirical clinical evaluation (which was included as a 'parameter' in the protocol, as a 'diplomatic concession) does not appear *a posteriori* justified by any fact.

In the present study however as in previous studies, no clear-cut difference in long-term evolution differentiates the patients. When summing up the results of 88 patients treated during the last 4 years, 22 of 42 patients retrospectively assigned to class A (relatively good prognosis) and 29 of the 46 patients assigned to class B died before 18 months of follow-up Although the mean life-span is higher in the first group the general prognosis remains severe in both groups.

When setting up a new protocol it will be important to take into consideration the possibility of obtaining complementary prognostic parameters, useful at least for evaluating the efficacy of the treatment.

Validity of the Treatment used

As most of the patients were assigned to group A (androgen therapy vs. symptomatic treatment alone), we can only give objective information for this group

14 patients were treated with androgens, of these patient 6 died (4 leukemias, 1 infection, 1 unknown cause) 10 received symptomatic treatment alone 5 of these patients died (2 leukemias,

2 cancers, 1 infection). According to the duration of the follow-up and the initial parameters of severity there is no difference between the two groups. It becomes more and more clear that androgens do not improve refractory anemia with an excess of bone marrow blasts. In the present series, anemia improved in only 1 patient.

Only 6 patients were treated with the suggested chemotherapy: 5 died, but retrospective analysis demonstrates that in these 5 patients, death occurred either early in the evolution or chemotherapy was not correctly used; only 1 case received the scheduled monthly courses during the 12 months of treatment and remains alive, without any improvement, however (number of blasts, pancytopenia, need for transfusions, CFC number).

Conclusions and Suggestion

The results of our cooperative study suggest that: prospective evaluation founded only on the number of blasts and the empirical evaluation of severity is insufficient; the severity of the disease is high, whatever the initial parameters, median life-span is less than 1 year with more than 60% of the patients dying from acute leukemia. It then appears ethical to consider the systematic use of chemotherapy in all the cases. A new protocol, founded on these data, could be discussed.

Dr Y. N. Jean, Département de médecine nucléaire, Hôpital St-Louis,
F 75010 Paris (France)

The second interesting information concerns the evolution in the three therapeutic weapons: the actuarial survival curves are absolutely similar in the three groups, which indicates that neither androgen therapy nor cytosine-arabinoside at the dosage used do modify the prognosis. On the other hand, the cause of death was similar in each group (7 deaths from leukemia in the androgen-treated group, 9 in the nontreated group 7 in the cytosine-arabinoside-treated group), which indicates that the chemotherapy used is inadequate (or insufficient) and that androgen therapy does not accelerate blast proliferation, as it has been suggested.

The detailed results will be published elsewhere.

Preliminary Results of the Study in P ogress

The protocol of this study in its final version (July 1977) integrates the previous data and suggests offering different therapeutic ways according to the *expected severity of the disease*, – *complet*ing our information on the possible efficacy of androgen therapy and trying to define the possible clinical interest of specific antileukemic treatment.

From the initial evaluation and a 3-month survey without specific therapy it was expected that it could be possible to discriminate between two groups of patients. group A with medullary blastosis constantly below 20%, without blood invasion by blasts and without clinical symptoms of progress, and group B, with an increasing percentage of blasts, appearance of circulating blastosis or clinical symptoms of progress. Group A patients had to be followed, according to randomization, on androgen therapy or symptomatic treatment only (with the possibility of joining group B at any time during the survey) group B patients were treated by monthly chemotherapy regimens vincristine (1.5 mg/m²) on the 1st day hydroxyurea (0.6 mg/m²) every 12 h for 5 days.

At the present time (January 1979) complete data on 30 cases only are available with a follow up of 6 months or more. The following preliminary conclusions can be drawn.

Validity of an a priori *Classification of the Patients*

Only 6 patients were assigned to group B (severe prognosis, chemotherapy) 24 to class A. When revising the charts, it was clear that a large fraction of the latter cases exhibited objective class B characteristics (13 of the 24 cases) It is difficult to understand why so many severe cases were considered by their physician as relatively mild one explanation could be the fear of using 'aggressive chemotherapy including monthly hospitalization and compelling supervision, on the other hand, it is generally assumed (without objective proof) that chemotherapy in this anemia is useless and often noxious in old patients (9 of these 13 patients were more than 70 years old).

It is certainly difficult to define clear prognostic criteria. Our data, however suggest relatively clear parameters: initially high (more than 20%) or an increasing percentage of blasts in the bone marrow eventually the presence of blasts in the circulation, and the degree of granulocytopenia have been previously clearly defined as unfavorable findings other objective parameters, as cell kinetics (bone marrow autoradiography and Fe kinetics), quantification of bone marrow stem cells and karyotypic studies, are used only in a limited number of cases, empirical clinical evaluation (which was included as a 'parameter' in the protocol, as a diplomatic' concession) does not appear *a posteriori* justified by any fact.

In the present study however as in previous studies, no clear-cut difference in long-term evolution differentiates the patients. When summing up the results of 88 patients treated during the last 4 years, 22 of 42 patients retrospectively assigned to class A (relatively good prognosis) and 29 of the 46 patients assigned to class B died before 18 months of follow-up Although the mean life-span is higher in the first group the general prognosis remains severe in both groups.

When setting up a new protocol, it will be important to take into consideration the possibility of obtaining complementary prognostic parameters, useful at least for evaluating the efficacy of the treatment.

V lidity of the Treatment used

As most of the patients were assigned to group A (androgen therapy vs. symptomatic treatment alone), we can only give objective information for this group

14 patients were treated with androgens, of these patients 6 died (4 leukemias, 1 infection, 1 unknown cause) 10 received symptomatic treatment alone 5 of these patients died (2 leukemias,

2 cancers, 1 infection) According to the d m of the follow-up and the initial parameters erity there is no difference between the groups. It becomes more and more clear that drugs do not improve refractory anemia with excess of bone marrow blasts. In the present series, anemia improved in only 1 patient.

Only 6 patients were treated with the suggested chemotherapy: 5 died, but retrospective nalysis demonstrates that in these 5 patients, death occurred either early in the evolution or chemotherapy was not correctly used, only 1 case received the scheduled monthly courses during the 12 months of treatment and remains alive, without any improvement, however (number f blasts, pancytopenia, need for transfusions, CFC number)

ons and ....

I sults of our cooperati study suggest t r pect aluation founded only on the mber f blast nd the empirical evaluation of severity is insufficient, the severity of the disease high hatever the initial parameters, median lif span is less than 1 year with more than 60° of the patient dying from acute leukemia. It then appears ethical to consider the systematic use of chemotherapy in all the cases. A new protocol, founded on these data, could be discussed.

Dr Y Najean, Département de médecine nucléaire Hôpital St-Louis,
F 75010 Paris (France)

Acta haemat. 61 330–333 (1979)

^{111}In Chloride Bone Marrow Scintigraphy and Ferrokinetic Studies in a Case of Sickle Cell Anemia with Transient Erythroid Aplasia[1]

Richard E Helmer III Bettye A Sayle Jack B Alperin and Frank H Gardner

Department of Internal Medicine (Division of Hematology-Oncology), Department of Radiology (Division of Nuclear Medicine) and Department of Internal Medicine, University of Texas Medical Branch, Galveston, Tex.

Key Words. Indium chloride scintigraphy Sickle cell anemia Erythropoietic aplastic crisis

Abstract. A patient with sickle cell anemia was hospitalized because of an acute erythropoietic aplastic crisis. ^{111}In chloride bone marrow scintigraphy performed during the aplastic crisis showed markedly impaired uptake of the radionuclide and simultaneous ^{59}Fe clearance $T_{½}$ studies were prolonged (420 9 min) Following recovery from the aplastic crisis ^{111}In scintigraphy revealed an expanded marrow and the radioiron clearance $T_{½}$ was accelerated (20 9 min) The clinical course, hemograms and bone marrow aspirations correlated with marrow scintigraphy ^{111}In appears to be a valuable bone marrow scanning agent for assessing the presence of intramedulllary erythropoietic activity

Introduction

A convenient radiopharmaceutical for bone marrow scintigraphy is needed which will reliably delineate the erythroid compartment. Radiocolloids label marrow reticuloendothelial (RE) cells, but RE activity does not always correspond with areas of erythropoiesis [6 12] the discrepancy is even more likely following chemotherapy or radiotherapy [3 6 8] ^{59}Fe would be a logical agent, but it is expensive and its short $T_{½}$ of 8 h precludes its use in most medical centers [6 10 12] Investigators have searched for other radionuclides more available than ^{59}Fe which retain its specificity for the erythron. ^{111}In appears to be the best agent now available

Indium binds *in vitro* and *in vivo* with transferrin and the plasma disappearance $T_{½}$ approaches 10 h [4 5 10] By 24 h radioactivity is detected within the skeleton in a pattern that parallels the expected marrow distribution [4] Reticulocyte-rich red cells incubated with transferrin-bound ^{111}In chloride or ^{59}Fe chloride have shown intracellular transport of both ions [6] following

[1] This investigation was supported in part by grant No. 5R01 CA 19997-02 awarded by the National Cancer Institute, DHEW

chromatography both ions have been identified within the hemoglobin peak [2] In may replace the iron molecule in the porphyrin ring of hemoglobin although definite evidence is lacking [6] The amount of In incorporated into erythrocytes appears related to the degree of erythropoiesis [2, 11]

Various reports have noted the efficacy of In marrow scintigraphy in such varied hematological conditions as pure red blood cell aplasia [1] lymphoma [3] myelofibrosis [8] and aplastic anemia [9] This report describes In bone marrow scintigraphy and ferrokinetic studies in a patient with sickle cell anemia during an aplastic crisis and following recovery



Fig. 1 Indium chloride marrow scintigraphy (posterior view). A during the erythropoietic crisis there is an absence of activity within the axial skeleton. Background, hepatic, and renal activity are noted. B following recovery normal axial marrow activity and peripheral extension are seen.

Case Study

A 20-year-old woman with sickle cell anemia was hospitalized following 3-day history of fever and severe headaches. When seen as an outpatient 1 week prior to admission she was asymptomatic and was taking daily supplemental folic acid PCV measurements as an outpatient generally have ranged from 0 21 to 0.25 The family history was noteworthy in that 2 siblings with sickle cell anemia had been in this hospital within the preceding 2 weeks for treatment of aplastic crises

Physical examination was not remarkable aside from an oral temperature of 38 4 °C and marked pallor The admitting hemogram showed Hb 4.9 g/dl, PCV 0 149 WBC 6.1 10^9/l (42% neutrophils, 2% bands, 42% lymphocytes, 5% basophils, 9% monocytes), and platelets 331.5 × 10^9/l. N reticulocytes were found on several blood smears Serum and whole blood folate levels were normal Serum iron measured 185 μg/dl and the total iron-binding capacity measured 198 μg/dl (93% saturation) Bone marrow aspiration revealed normal cellularity and severe erythroid hypoplasia with only 'giant pronormoblasts and scattered pronormoblasts present.

With the patient' consent, I marrow scintigraphy and ferrokinetic studies were performed. She received In chloride (Medi-Physics, Inc., Emeryville, Calif.) on the afternoon of admission and scintigraphy was performed 24 h later (fig. 1A). In this and the subsequent study I was injected without incubation in compatible plasma [8]. Ferrokinetic studies revealed an iron clearance T of 420 9 min (normal 60–120 min) on the 2nd hospital day Surface counting revealed no uptake of radioactivity over the sacrum or right knee. Counting over the liver revealed uptake but no release of radioactivity A repeat marrow aspiration 2 days later showed increased cellularity erythroid hyperplasia, and the absence of giant pronormoblasts. By the 9th hospital day the patient had reticulocytosis, her peripheral counts were stable (PCV 0.24), and she was discharged.

Table I. Summary of laboratory values

	PCV	Reticulocytes %	WBC $\times 10^9$/l	Platelets $\times 10^9$/l	Iron clearance $T_{1/2}$ min
Aplastic crisis	0.149	0	6.1	331.5	420.9
Recovery	0.24	12.6	15.3	528.5	20.9

Admission was required 3 months later for treatment of pleuritis. Physical examination revealed an oral temperature of 37.8 C and coarse rales over the right posterior chest. The hemogram showed a Hb 8.2 g/l, PCV 0.24 WBC 15.3 × 10^9/l (59% neutrophils, 3% bands, 1% eosinophils, 33% lymphocytes, 4% monocytes), platelets 528.5 × 10^9/l and reticulocyte count 12.6%. With the patient's consent, ^{111}In scintigraphy and radioiron clearance studies were repeated. Normal activity in the axial skeleton with peripheral extension into the long bones of the lower extremities was present (fig. 1B). The plasma iron clearance $T_{1/2}$ measured 20.9 min (table I).

Discussion

During her two hospitalizations the patient presented the opportunity to correlate erythropoiesis with ^{111}In scintigraphy. Initial bone marrow scintigraphy performed when there was a severe erythroid hypoplasia *showed almost no marrow uptake of the isotope*. Scintigraphy performed when there was a reticulocytosis indicating marrow erythroid hyperplasia showed a peripheral marrow extension similar to that reported in another patient with sickle cell anemia [6]. The hemograms and ^{59}Fe clearance studies before and following recovery correlate with the scintigraphic changes observed. External surface counting over the sacrum and the knee recorded the absence of erythropoietic activity during the aplastic crisis.

From a scintigraphic standpoint, this patient is similar to an individual with pure red cell aplasia in whom ^{111}In marrow scintigraphy was performed before and during erythropoietic recovery [10]. ^{111}In scintigraphy in another patient with pure red cell aplasia (and thymoma) has also shown the absence of marrow uptake [11]. Our own experience with a patient with pure red cell aplasia (without thymoma) also demonstrated marked inhibition of marrow ^{111}In localization.

At the present time there appears to be no consensus concerning the role of ^{111}In scintigraphy in localization of the marrow erythropoietic elements. Justifiable concern has been voiced regarding this point and some workers have cautioned that ^{111}In may not be specific for the erythron [7, 10]. Like ^{59}Fe, ^{111}In is cyclotron produced. However its $T_{1/2}$ of 2.8 days makes it accessible to most hospitals. Although the precise site(s) of ^{111}In binding is not fully agreed upon, we conclude that ^{111}In marrow scintigraphy does appear to be a reliable alternative to ^{59}Fe scintigraphy.

References

1 Bunn, H., McNeil, B., Rosenthal, D. and Krantz, S.: Bone-marrow imaging in pure red blood cell aplasia. Archs intern. Med. *136*: 1169–1172 (1976).

2 Farrer, P., Saha, G. and Katz, M.: Further observations on the use of In-transferrin for the visualization of bone marrow in man. J. nucl. Med. *14*: 394–395 (1973).

3 Gilbert, E., Earle, J.; Glatstein, E.; Goris, M., Kaplan, H., and Kriss, J.: ^{111}Indium bone marrow scintigraphy as an aid in selecting marrow biopsy sites for the evaluation of marrow elements in patients with lymphoma. Cancer *38* 1560–1567 (1976).

4 Goodwin, D. Goode, R., Brown, L., and Imbornone, C.: In-labelled transferrin for the detection of tumors. Radiology *100*. 175–179 (1971).

5 Hosain, F. McIntyre, P. Poulose, K., Stern, H., and Wagner, H., jr.: Binding of trace amounts of ionic indium-113m to plasma transferrin. Clinica chim. Acta *24* 69–75 (1969).

6 Lilien, D., Berger, H.; Anderson, D., and Bennett, L.: ^{111}In-chloride: a new agent for bone marrow imaging. J. nucl. Med. *14* 184–186 (1973).

7 McIntyre, P.; Larson, S., Eikman, E., Colman, M., Scheffel, U., and Hodkinson, B.: Comparison of the metabolism of iron-labeled transferrin (Fe-TF) and indium-labeled transferrin (In-TF) by the erythropoietic marrow J. nucl. Med. *15* 856–862 (1974).

8 McNeil, B., Holman, B., Button, L., and Rosenthal, D.: Use of indium chloride scintigraphy in patients with myelofibrosis. J. nucl. Med. *15* 647–651 (1974).

9 McNeil, B., Rappeport, J. and Nathan, D.: Indium chloride scintigraphy: an index of severity in patients with aplastic anemia. Br. J. Haemat. *34* 599–604 (1976).

10 Merrick, M., Gordon-Smith, E., Lavender, J. and Szur, L.: A comparison of ^{111}I with ^{59}Fe and ^{99m}Tc-sulphur colloid for bone marrow scanning. J. nucl. Med. *16*. 66–68 (1975).

11 Staub, R. and Gaston, E.: ^{111}In-chloride distribution and kinetics in hematologic disease. J. nucl. Med. *14* 456–457 (1973).

12 Van Dyke, D.; Shkurkin, C.; Price, D., Yano, Y. and Anger, H.: Differences in distribution of erythropoietic and reticuloendothelial marrow in hematologic disease. Blood *30*: 364–374 (1967).

Frank H. Gardner MD
Division of Hematology-Oncology
MW-404 John Sealy Hospital,
University of Texas Medical Branch,
Galveston, TX 77550 (USA)

Table I. Summary of laboratory valuees

	PCV	Reticulocytes %	WBC $\times 10^9$/l	Platelets $\times 10^9$/l	Iron clearance $T_{1/2}$ min
Aplastic crisis	0.149	0	6.1	331.5	420.9
Recovery	*0.24*	*12.6*	*15.3*	*528.5*	*20.9*

Admission was required 3 months later for treatment of pleuritis. Physical examination revealed an oral temperature of 37.8 C and coarse rales over the right posterior chest. The hemogram showed a Hb 8.2 g/l, PCV 0.24 WBC 15.3 × 10⁹/l (59% neutrophils, 3% bands, 1% eosinophils, 33% lymphocytes, 4% monocytes), platelets 528.5 × 10⁹/l and reticulocyte count 12.6%. With the patient's consent, [1] In scintigraphy and radioiron clearance studies were repeated. Normal activity in the axial skeleton with peripheral extension into the long bones of the lower extremities was present (fig. 1B) The plasma iron clearance $T_{1/2}$, measured 20.9 min (table I).

Discussion

During her two hospitalizations, the patient presented the opportunity to correlate erythropoiesis with ¹¹¹In scintigraphy. Initial bone marrow scintigraphy performed when there was a severe erythroid hypoplasia showed almost no marrow uptake of the isotope. Scintigraphy performed when there was a reticulocytosis indicating marrow erythroid hyperplasia showed a peripheral marrow extension similar to that reported in another patient with sickle cell anemia [6]. The hemograms and ⁵⁹Fe clearance studies before and following recovery correlate with the scintigraphic changes observed. External surface counting over the sacrum and the knee recorded the absence of erythropoietic activity during the aplastic crisis.

From a scintigraphic standpoint, this patient is similar to an individual with pure red cell aplasia in whom ¹¹¹In marrow scintigraphy was performed before and during erythropoietic recovery [10]. ¹¹¹In scintigraphy in another patient with pure red cell aplasia (and thymoma) has also shown the absence of marrow uptake [11]. Our own experience with a patient with pure red cell aplasia (without thymoma) also demonstrated marked inhibition of marrow ¹¹¹In localization.

At the present time there appears to be no consensus concerning the role of ¹¹¹In scintigraphy in localization of the marrow erythropoietic elements. Justifiable concern has been voiced regarding this point and some workers have cautioned that ¹¹¹In may not be specific for the erythron [7, 10]. Like ⁵⁹Fe, ¹¹¹In is cyclotron produced. However its $T_{1/2}$ of 2.8 days makes it accessible to most hospitals. Although the precise site(s) of ¹¹¹In binding is not fully agreed upon, we conclude that ¹¹¹In marrow scintigraphy does appear to be a reliable alternative to ⁵⁹Fe scintigraphy.

References

1 Bunn, H.; McNeil, B.; Rosenthal, D., and Krantz, S.: Bone-marrow imaging in pure red blood cell aplasia. Archs intern. Med. 136: 1169–1172 (1976).

2 Farrer, P.; Saha, G., and Katz, M.: Further observations on the use of In-transferrin for the visualization of bone marrow in man. J. nucl. Med. 14: 394–395 (1973).

Methods

Venous blood for hemoglobin analysis was collected from each of 23 baboons of the species *Papio anubis*. Results of oral glucose tolerance tests were normal in each of the 15 (14 females and 1 male) baboons that composed the control group. The weights of these nondiabetic baboons varied from 10.9 to 21.0 kg (average weight = 15.2 kg). The other group included eight female baboons with pancreatectomy induced diabetes mellitus. The weights of these diabetic animals varied from 11.0 to 15.5 kg (average weight = 14.4 kg), and the duration of diabetes varied from 99 to 105 months (99.6 months average).

All diabetic baboons and three control baboons were individually caged in Galveston. The other nondiabetic baboons were held in gang cages in Oklahoma City. Both groups of animals received the same diet; however the diabetic group received supplemental vitamins, additional carbohydrate in the form of extra fresh fruit and candy and pancreatic enzymes. Diabetic baboons received daily injections of NPH insulin; nevertheless, each exhibited nonfasting preinsulin plasma glucose concentration ≥ 4.24 g/l at the time of blood collection.

Blood was also collected for hemoglobin analysis from two groups of patients. The control group included 26 women and 25 men all of whom exhibited fasting and 2-hour postprandial serum glucose level ≤ 1.20 g/l at the time of blood collection. The other group included 26 women and 15 men all of whom were known to have poorly regulated diabetes mellitus for more than 3 months. Each diabetic patient exhibited fasting plasma or serum glucose concentration ≥ 3.18 g/l when blood was collected for hemoglobin examination. All measurements of glucose in plasma or serum were performed by an automated hexokinase method.

Hemolysates prepared from anticoagulated baboon and human blood were chromatographed on 2 × 17.5 cm columns of polymethacrylic acid resin (Bio Rex 70, 200–400 mesh) as described by *Trivelli et al.* [19]. Using the same buffers, human and baboon hemolysates were also chromatographed on 2 × 5 cm columns of Bio Rex 70 as suggested by *Kynoch and Lehmann* [14]. Baboon hemolysates were further analyzed by electrophoresis on citrate agar plates, pH 6.0, and on cellulose acetate plates using Tris-EDTA borate buffer, pH 8.6 [17]. The percent of alkali resistent hemoglobin (Hb F) was also measured in each baboon hemolysate [2].

Results

Electrophoresis of each baboon hemolysate on citrate agar showed a single hemoglobin component with a mobility nearly identical to human Hb A. Electrophoresis of each baboon hemolysate on cellulose acetate showed a single hemoglobin component with a mobility between human Hb A and human Hb F. No minor hemoglobin component, similar to human Hb A_2 ($\alpha_2\delta_2$) was found in any of the baboon hemolysates. These observations are in keeping with the results of previous electrophoretic studies performed on blood from baboons of the species *Papio doguera (= anubis)* and other old world monkeys [10]. Mean Hb F values (± SEM) for nondiabetic and diabetic baboons were 0.58 ± 0.06 and 0.56 ± 0.08%, respectively ($p > 0.05$).

In studies to be described, the hemoglobin found in baboon hemolysates will be called baboon Hb A and is different from human Hb A. When either human or baboon hemolysates were chromatographed on 2 × 17 cm columns of Bio Rex 70 [19] three early eluting hemoglobin bands (designated A_{1a}, A_{1b}, and A_{1c}) were observed to separate from hemoglobin A_0. Neither baboon Hbs A_{1a} and A_{1b} nor human Hbs A_{1a} and A_{1b} completely separated from each other; therefore, they were eluted together [12, 19]. Hb A_{1c} (either baboon or human) was the second hemoglobin fraction to be eluted, and the remainder Hb A_0 became the last. When hemolysates were chromato-

Acta haemat. 61 334-338 (1979)

A Comparison of Hemoglobin A_{1c} in Human and Baboon Blood[1,2]

J B Alperin, P A Dow and L. C Stout

Departments of Internal Medicine (Division of Hematology-Oncology), Human Biological Chemistry and Genetics, and Pathology University of Texas Medical Branch, Galveston, Tex.

Key Words. Diabetes mellitus Glycosylated hemoglobin Hb A_{1c} *Papio anubis*

Abstract. Cation exchange chromatography was performed on hemolysates prepared from human and baboon *(Papio anubis)* blood. In humans with diabetes mellitus and in baboons with pancreatectomy induced diabetes mellitus there were significant increases in Hbs A_1, A_{1c}, and $A_{1a}+A_{1b}$. The quantity of Hb A_1 and Hb A_1 in nondiabetic baboons was approximately one half the quantity of Hb A_1 and Hb A_{1c} in nondiabetic humans. These differences may be explained by the differences in survival time between human and baboon red cells.

Introduction

Cation exchange chromatography of hemolysates prepared from normal human blood reveal several minor hemoglobins that are eluted before Hb A_0, the major hemoglobin component [9] These early eluting hemoglobins, collectively called Hb A_1 represent posttranslational modifications of normal adult hemoglobin (Hb A $\alpha_2\beta_2$). Hb A_{1c}, the most abundant of these minor hemoglobins, is present in concentrations 2–3 times normal in patients with poorly regulated diabetes mellitus, and the concentration of Hb A_{1c} in blood may be correlated with the time averaged blood glucose level over a period of weeks [5 13 19] Other A_1 hemoglobins are also increased in the blood of patients with diabetes mellitus [3 7 13] Elevated levels of Hb A_{1c} have been observed in the blood of mice with diabetes mellitus [12] This report describes results of quantitating A_1 hemoglobins in the blood of normal and diabetic baboons. The results are compared with measurements of A_1 hemoglobins in normal and diabetic humans.

1 The diabetic baboons were supported by a grant from the John A. Hartford Foundation, Inc. The normal baboons are from the colony of Dr *Warren M Crosby* University of Oklahoma Health Sciences Center Oklahoma City Okla.

2 Some of the human blood used in this study was obtained from patients at the Clinical Research Center University of Texas Medical Branch under support of DHEW grant RR 73, Division of Research Resources, National Institutes of Health.

in normal humans. Since plasma glucose concentrations are comparable in both non-diabetic baboons and nondiabetic humans, these differences in percent Hb A_I and Hb A_{Ic} probably reflect the observed differences in red cell life-span between man (100–120 days) and baboons (30–60 days) [1 10] Similar differences are noted when Hb A_{Ic} levels in man are compared to Hb A_{Ic} levels in mice [12] since the life span of mouse erythrocytes is nearly 40 days [6]

Studies performed on hemolysates prepared from human blood show that Hb A_{Ic} cochromatographs with Hb F and false elevations of *Hb A_{Ic}* have been observed in patients with increased amounts of Hb F [5] In this study each baboon had a Hb F level within the previously described range of Hb F in baboons [10] and mean Hb F levels in nondiabetic and diabetic baboons were not significantly different. Thus, the increased Hb A_{Ic} observed in diabetic baboons was not due to elevations of Hb F

Data presented in table I also indicate that measuring Hb A_I by chromatography on a short (2 × 5 cm) column of Bio Rex 70 is an accurate indicator of poorly regulated diabetes mellitus in baboons just as it is in man [14] This is because in human and baboon blood, Hb A_{Ic} comprises more than 60% of all A_I hemoglobins. *Flückiger and Winterhalter* [8] have described a different technique for measuring Hb A_{Ic} which depends on the release of 5-hydroxymethyl furfural from hemoglobin and subsequent color development with 2-thiobarbituric acid. This method may replace chromatography for serial measurements of Hb A_I (or Hb A_{Ic}) to follow the course of diabetes mellitus.

Data presented in table I show that the sum of Hbs A_{Ia} + A_{Ib} is significantly greater in diabetic than non-diabetic baboons just as the sum of Hbs A_{Ia} + A_{Ib} is significantly greater in diabetic than nondiabetic humans [3 5 7 16] Human Hbs A_{Ia} and A_{Ib} are also examples of posttranslational modifications of Hb A [5] Working with human blood and using a high resolution chromatographic system, *McDonald et al.* [16] have observed complete separation of Hb A_{Ia} from Hb A_{Ib} so that these hemoglobins can be eluted independently of each other Furthermore, these investigators observed that Hb A_{Ia} is composed of two components, Hb A_{Ia1} and Hb A_{Ia2}. The proposed structures for *Hb A_{Ia1}* and *Hb A_{Ia2}* are 1,6-diphosphofructose hemoglobin and glucose-6-phosphate hemoglobin, respectively [3]. The structure of Hb A_{Ib} has not been ascertained, but preliminary studies suggest this is also a glycosylated hemoglobin [3 16] Hb A_{Ia1} and Hb A_{Ia2} are present in normal amounts in patients with poorly regulated diabetes mellitus however, Hb A_{Ib} is increase in uncontrolled diabetics [3 16] Because of the great structural similarity between human Hb A and baboon Hb A [15 18] the A hemoglobins observed in baboons probably have the same structures as human A hemoglobins.

References

1 Berlin, N. L. Laboratory evaluation of erythrokinetics; in Williams, Beutler, Erslev and Rundles, Hematology 2nd ed., pp. 237–246 (McGraw-Hill, New York 1977).

2 Betke, K., Marti, H. R., and Schlicht, L. Estimation of small percentages of foetal haemoglobin. Nature, Lond. *184* 1877–1878 (1959).

3 Bunn, H. F Gabbay K. H., and Gallop, P M.. The glycosylation of hemoglobin: rele

Table I

	Percent Hb A_{1a} + A_{1b}[1]	Percent Hb A_{1c}[1]	Percent Hb A_1[2]
Baboon, control (15)	1.53 ± 0.08	2.40 ± 0.40	3.52 ± 0.48
Baboon, diabetic (8)	2.63 ± 0.21	5.86 ± 0.85	8.55 ± 1.03
p value	< 0.01	< 0.01	< 0.01
Human, control (51)	1.67 ± 0.18	4.10 ± 0.28	6.13 ± 0.34
Human, diabetic (41)	2.79 ± 0.26	11.10 ± 0.52	14.10 ± 0.58
p value	< 0.01	< 0.01	< 0.01

All data expressed as mean ± SEM and analyzed by the Student's t test for paired data. Numerals in parentheses indicate the number of baboons or humans in each group.

[1] Eluted from 2 × 17 cm columns of Bio Rex 70 [19].

[2] Eluted from 2 × 5 cm columns of Bio Rex 70 [14].

graphed on 2 × 5 cm columns of Bio Rex 70 [14] both baboons and humans exhibited only one early eluting hemoglobin component, Hb A_1.

Data presented in table I show that in both baboons and man Hb A_{1c} represents the major early eluting hemoglobin component (i.e. over 60% of the A_1 hemoglobins). This table also shows that baboons with poorly regulated diabetes mellitus, just as humans with poorly regulated diabetes, have significantly elevated values for Hb A_1, Hb A_{1c}, and Hbs A_{1a} + A_{1b}.

Discussion

Studies performed in man show that synthesis of Hb A_{1c} results from the nonenzymatic union of glucose and Hb A. The adduct thus formed has an aldimine structure (Schiff base) which immediately undergoes molecular rearrangement to form a ketoamine linkage [4, 16]. The ultimate structure is then 1-amino 1-deoxyfructose linked to the NH_2 terminal (valyl) end of each hemoglobin β chain [11]. Studies performed on humans and mice indicate that synthesis of Hb A_{1c} is a continuous process throughout the life-span of erythrocytes so that old red cells have significantly more Hb A_1 than young red cells [5, 7, 12]. At least two factors govern the quantity of Hb A_{1c} in blood, erythrocyte life-span and the time-averaged blood glucose concentration over a period of weeks [5, 13, 19]. In poorly regulated diabetes mellitus, Hb A_{1c} levels are several times greater than Hb A_{1c} levels in non-diabetics [5, 7, 13], whereas Hb A_{1c} levels tend to be reduced in the presence of hemolytic anemia [5].

Data presented in table I show the average Hb A_1 level in baboons with pancreatectomy induced diabetes mellitus is 2–3 times greater than the average Hb A_{1c} in nondiabetic baboons. This is similar to differences in Hb A_1 levels between normal and diabetic humans as indicated in table I and as reported by others [5, 13, 19]. It is notable that the mean levels of Hb A_1 and Hb A_{1c} in normal baboons are approximately one-half the mean Hb A_1 and Hb A_1 levels

in normal humans. Since plasma glucose concentrations are comparable in both nondiabetic baboons and nondiabetic humans, these differences in percent Hb A_1 and Hb A_{1c} probably reflect the observed differences in red cell life-span between man (100–120 days) and baboons (30–60 days) [1, 10]. Similar differences are noted when Hb A_{1c} levels in man are compared to Hb A_{1c} levels in mice [12] since the life span of mouse erythrocytes is nearly 40 days [6].

Studies performed on hemolysates prepared from human blood show that Hb A_{1c} cochromatographs with Hb F and false elevations of Hb A_{1c} have been observed in patients with increased amounts of Hb F [5]. In this study each baboon had a Hb F level within the previously described range of Hb F in baboons [10] and mean Hb F levels in nondiabetic and diabetic baboons were not significantly different. Thus, the increased Hb A_{1c} observed in diabetic baboons was not due to elevations of Hb F.

Data presented in table I also indicate that measuring Hb A_1 by chromatography on a short (2 × 5 cm) column of Bio Rex 70 is an accurate indicator of poorly regulated diabetes mellitus in baboons just as it is in man [14]. This is because in human and baboon blood, Hb A_{1c} comprises more than 60% of all A_1 hemoglobins. *Flückiger and Winterhalter* [8] have described a different technique for measuring Hb A_{1c} which depends on the release of 5-hydroxymethyl furfural from hemoglobin and subsequent color development with 2-thiobarbituric acid. This method may replace chromatography for serial measurements of Hb A_1 (or Hb A_{1c}) to follow the course of diabetes mellitus.

Data presented in table I show that the sum of Hbs $A_{1a} + A_{1b}$ is significantly greater in diabetic than non-diabetic baboons just as the sum of Hbs $A_{1a} + A_{1b}$ is significantly greater in diabetic than nondiabetic humans [3, 5, 7, 16]. Human Hbs A_{1a} and A_{1b} are also examples of posttranslational modifications of Hb A [5]. Working with human blood and using a high resolution chromatographic system, *McDonald et al.* [16] have observed complete separation of Hb A_{1a} from Hb A_{1b} so that these hemoglobins can be eluted independently of each other. Furthermore, these investigators observed that Hb A_{1a} is composed of two components, Hb A_{1a1} and Hb A_{1a2}. The proposed structures for Hb A_{1a1} and Hb A_{1a2} are 1,6-diphosphofructose hemoglobin and glucose-6-phosphate hemoglobin, respectively [3]. The structure of Hb A_{1b} has not been ascertained, but preliminary studies suggest this is also a glycosylated hemoglobin [3, 16]. Hb A_{1a1} and Hb A_{1a2} are present in normal amounts in patients with poorly regulated diabetes mellitus, however Hb A_{1b} is increase in uncontrolled diabetics [3, 16]. Because of the great structural similarity between human Hb A and baboon Hb A [15, 18], the A_1 hemoglobins observed in baboons probably have the same structures as human A_1 hemoglobins.

References

1 Berlin, N. L.: Laboratory evaluation of erythrokinetics; in Williams, Beutler, Erslev and Rundles, Hematology; 2nd ed., pp. 237–246 (McGraw-Hill, New York 1977).

2 Betke, K., Marti, H. R., and Schlicht, I.: Estimation of small percentages of foetal haemoglobin. Nature, Lond. *184*: 1877–1878 (1959).

3 Bunn, H. F.; Gabbay, K. H., and Gallop, P. M.: The glycosylation of hemoglobin: rele-

vance to diabetes mellitus. Science 200 21–27 (1978)
4 Bunn, H. F Haney D N., Gabbay K. H., and Gallop, P M.. Further identification of the nature and linkage of the carbohydrate in hemoglobin A. Biochem. biophys. Res. Commun. 67 103–109 (1975)
5 Bunn, H. F., Haney D N., Kamin, S., Gabbay K. H., and Gallop P M.. The biosynthesis of human hemoglobin A_{1c}. Slow glycosylation of hemoglobin *in vivo* J clin. Invest. 57 1652–1659 (1976)
6 Burwell, E. L., Brinkley B. A., and Finch, C. A.. Erythrocyte life span in small animals. Comparison of two methods employing radioiron. Am. J Physiol. 172 718–724 (1953).
7 Fitzgibbons, J F Koler R. D and Jones, R. T.. Red cell age-related changes of hemoglobins A_1 and A_{1c} in normal and diabetic subjects. J clin. Invest. 58 820–824 (1976).
8 Flückiger R. and Winterhalter K. H.. *In vitro* synthesis of hemoglobin A_{1c}. FEBS Lett. 71 356–360 (1976).
9 Huisman, T H. J and Meyering, C. A.. Studies on the heterogeneity of different human hemoglobin types in carboxymethylcellulose and in Amberlite IRC 50 chromatography Qualitative aspects. Clinica chim. Acta 5 103–123 (1960).
10 Huser H. J.. Atlas of comparative primate hematology pp. 56–85 (Academic Press, New York 1970)
11 Koenig, R. J., Blobstein, S. H., and Cerami, A.. Structure of carbohydrate of hemoglobin A. J biol. Chem. 252 2992–2997 (1977).
12 Koenig, R. J and Cerami, A.. Synthesis of hemoglobin A in normal and diabetic mice: potential model of basement membrane thickening. Proc. natn. Acad. Sci. USA 72. 3687–3691 (1975)
13 Koenig, R. J., Peterson, C. M., Saudek, C. Lehrman, M., and C Correlation of glucose regulation globin A_{1c} in diabetes mellitus. New Med. 295 417–420 (1976)
14 Kynoch, P A. M and Lehmann, H. timation ($2^1/_2$ hours) of glycosylated bin for routine purposes. Lancet ii. 16 (1
15 Lehmann, H. and Huntsmann, R. G. haemoglobins, 2nd ed., p. 65 (P adelphia 1974).
16 McDonald, M. J., Shapiro, R. Solway J and Bunn, H. F.. Glycosylate nor components of human adult hemog Purification, identification, and partial tural analysis. J biol. Chem. 253 2327 (1978).
17 Schneider R. G., Hightower T S., and T S.. Abnormal hemoglobins in a quarter lion people. Blood 48 629–639 (1976).
18 Sullivan, B., Bonaventura, J., and Nute, P E.. Structure and function of boon hemoglobins; in Goodman, Tashian, Tashian, Molecular anthropology pp. 22 (Plenum Press, New York 1976).
19 Trivelli, L. A., Ranney H. M., and Lai, H. Hemoglobin components in patients with betes mellitus. New Engl. J Med. 353–357 (1971).

Received, July 17 1978
Accepted, December 8, 1978

Jack B. Alperin, MD, 422 Clinical Sciences Building, University of Texas Medical Branch Galveston, TX 77550 (USA)

Correspondence

Acta haemat. 61: 339 (1979)

To the Editor

Sir

In the discussion part of their interesting paper on β-Thalassemia in Sicily' *Schilirò et al.* [Acta haemat. 60 193 (1978)] stated that *Friedman et al.* [1] have recently described patients with genetic, clinical and hematological pictures identical to those of *our patients with Rietti-Greppi-Micheli disease* (RGMD), in whom imbalance of β-globin chain synthesis is similar to our subjects. Although genetic and clinical pictures of RGMD in the patients referred to in the article, are similar to the patients studied by us [1] the following points should be brought to attention: In our cases the globin ratios (β/α) in the peripheral blood were not different from those of heterozygous β-thalassemia, but in the patients of *Schilirò et al.* with RGMD they were significantly different ($p < 0.01$). In addition, bone marrow globin chain ratios of our cases were studied and they were *found to be different from* those of homozygous and heterozygous β-thalassemias and the free α-chain pools in the marrow were found elevated these studies were not done in their patients with RGMD Peripheral blood globin chain ratios alone are enough to indicate that RGMD is neither identical *nor similar to* the patients described by us.

Reference

1 Friedman, S. H.; Ozsoylu, S., Luddy R., and Schwartz, E. Heterozygous beta-thalassemia of unusual severity Br J Haemat. 32. 65–77 (1976).

Sinasi Ozsoylu, MD, Children's Medical Center, Hacettepe University Ankara (Turkey)

M Blombäck (ed.)
Synthetic Substrates and Synthetic Inhibitors
The Use of Chromogenic Substrates in Studies of Haemostatic Mechanism
Haemostasis, vol. 7 No. 2–3, 1978
Karger Basel 1978
138 pp., SFr 38.–
ISBN 3-8055-2907-4

This double issue of Haemostasis No 2–3 (1978) is entirely dedicated to the new application of synthetic substrates and inhibitors in the field of blood coagulation, fibrinolysis and related problems. The different articles have been presented at a side symposium of the 6th International Congress of the International Society on Haemostasis and Thrombosis, Philadelphia 1977 After an introduction on the search for new chromogenic substrates most papers are aimed at the determination of clotting factors, prekallikrein and inhibitors. Other papers are concerned with the specificity of the substrates and problems arising from their generalized use.

The chromogenic substrates are an extremely interesting tool for the detection and quantitative assay of proteases, among them clotting and fibrinolysis factors. The synthetic inhibitors of proteases presented in two papers are a valuable complement. This issue of *Haemostasis* gives a wide information and can be recommended to all interested in chromogenic substrates and synthetic inhibitor

Prof. Dr F Duckert, Basel

J O Jost und H Knoche
Leitfaden der Hämatologie und Blutgruppenserologie
Fischer Stuttgart 1977 XII + 147 pp DM 19.80
ISBN 3-437-00246-5

Dieser kleine Leitfaden soll eine Doppelfunktion erfüllen, welcher er nicht in allen Belangen gerecht wird: Der Text entspringt einem hämatologischen Einführungskurs für medizinisch-technische Assistenten, für welche er auch geeignet ist. Im Hämatologielabor verwendete Techniken werden im Detail beschrieben und in ihrer Funktionsweise verständlich erklärt. Daneben enthält das Büchlein aber zusätzliches Grundlagenwissen, das z. T die Bedürfnisse der medizinischen Laboranten übersteigt. Für Medizinstudenten, für welche der Leitfaden ebenfalls bestimmt sein soll, ist aber eben dieses Grundlagenwissen zu wenig umfassend und teilweise lückenhaft dargestellt. Verständlicherweise würde eine eingehende klinische Beschreibung hämatologischer Krankheitsbilder den Rahmen des Werkes sprengen, aus den vorliegenden klinischen Kurzdarstellungen können aber Medizinstudenten kaum grossen Nutzen ziehen.

Der Inhalt des Büchleins ist offensichtlich auf spezifisch deutsche Verhältnisse abgestimmt. Unseres Erachtens ist er in einigen Teilen zu ausführlich, in anderen Teilen zu summarisch. Wichtige Techniken wie diejenige des Knochenmarksausstriches sind ungenügend diskutiert. Die für das Laboratorium wichtige morphologische Beschreibung der verschiedenen Zellsysteme kommt leider etwas zu kurz, und das Buch enthält nur wenige, eher schematische Abbildungen von Blutzellen. Diese Lücke kann auch durch elektronen-mikroskopische Bilder nicht geschlossen werden. Wichtige Details wie die Bedeutung und der Nachweis von Innenkörpern, Siderozyten und Sideroblasten werden nur am Rande oder gar nicht erwähnt. Die Abschnitte über Blutgruppenserologie und Blutgerinnung sind in vielen Belangen zu summarisch und ungenau abgehandelt. So wird z. B. für die Unterscheidung von Rhesus- und ABO-Inkompatibilität die Verwendung des Coombs-Tests statt der aussagekräftigen Antikörperbestimmung empfohlen. Auch der systematische Aufbau des Stoffes ist nicht in jeder Hinsicht befriedigend.

Alles in allem kann sich der kleine Leitfaden mit Vorbehalt als Lehrmittel für technische Laborkurse eignen, für den Medizinstudenten können wir ihn aber nicht empfehlen.

M Frey-Wettstein, Zürich

Author Index

(B) = Book Reviews

Book Reviews

M. Blombäck (ed.)
Synthetic Substrates and Synthetic Inhibitors
The Use of Chromogenic Substrates in Studies of Haemostatic Mechanism
Haemostasis, vol. 7 No. 2–3, 1978
Karger Basel 1978
138 pp SFr 38.–
ISBN 3-8055-2907-4

This double issue of Haemostasis No. 2–3 (1978) is entirely dedicated to the new application of synthetic substrates and inhibitors in the field of blood coagulation, fibrinolysis and related problems. The different articles have been presented at a side symposium of the 6th International Congress of the International Society on Haemostasis and Thrombosis, Philadelphia 1977. After an introduction on the search for new chromogenic substrates most papers are aimed at the determination of clotting factors, prekallikrein and inhibitors. Other papers are concerned with the specificity of the substrates and problems arising from their generalized use.

The chromogenic substrates are an extremely interesting tool for the detection and quantitative assay of proteases, among them clotting and fibrinolysis factors. The synthetic inhibitors of proteases presented in two papers are a valuable complement. This issue of *Haemostasis* gives a wide information and can be recommended to all interested in chromogenic substrates and synthetic inhibitor.

Prof. Dr. F. Duckert, Basel

J. O. Jost und *H. Knoche*
Leitfaden der Hämatologie und Blutgruppenserologie
Fischer Stuttgart 1977 XII + 147 pp., DM 19.80
ISBN 3-437-00246-5

Dieser kleine Leitfaden soll eine Doppelfunktion erfüllen, welcher er nicht in allen Belangen gerecht wird. Der Text entspringt einem hämatologischen Einführungskurs für medizinisch-technische Assistenten, für welche er auch geeignet ist. Im Hämatologielabor verwendete Techniken werden im Detail beschrieben und in ihrer Funktionsweise verständlich erklärt. Daneben enthält das Büchlein aber zusätzliches Grundlagenwissen, das z. T. die Bedürfnisse der medizinischen Laboranten übersteigt. Für Medizinstudenten, für welche der Leitfaden ebenfalls bestimmt sein soll, ist aber eben dieses Grundlagenwissen zu wenig umfassend und teilweise lückenhaft dargestellt. Verständlicherweise würde eine eingehende klinische Beschreibung hämatologischer Krankheitsbilder den Rahmen des Werkes sprengen, aus den vorliegenden klinischen Kurzdarstellungen können aber Medizinstudenten kaum grossen Nutzen ziehen.

Der Inhalt des Büchleins ist offensichtlich auf spezifisch deutsche Verhältnisse abgestimmt. Unseres Erachtens ist er in einigen Teilen zu ausführlich, in anderen Teilen zu summarisch. Wichtige Techniken wie diejenige des Knochenmarksausstriches sind ungenügend diskutiert. Die für das Laboratorium wichtige morphologische Beschreibung der verschiedenen Zellsysteme kommt leider etwas zu kurz, und das Buch enthält nur wenige, eher schematische Abbildungen von Blutzellen. Diese Lücke kann auch durch elektronen-mikroskopische Bilder nicht geschlossen werden. Wichtige Details wie die Bedeutung und der Nachweis von Innenkörpern, Siderozyten und Sideroblasten werden nur am Rande oder gar nicht erwähnt. Die Abschnitte über Blutgruppenserologie und Blutgerinnung sind in vielen Belangen zu summarisch und ungenau abgehandelt. So wird z. B. für die Unterscheidung von Rhesus- und ABO-Inkompatibilität die Verwendung des Coombs-Tests statt der aussagekräftigen Antikörperbestimmung empfohlen. Auch der systematische Aufbau des Stoffes ist nicht in jeder Hinsicht befriedigend.

Alles in allem kann sich der kleine Leitfaden mit Vorbehalt als Lehrmittel für technische Laborkurse eignen, für den Medizinstudenten können wir ihn aber nicht empfehlen.

M. Frey-Wettstein, Zürich

Author Index

(B) = Book Reviews

Subject Index

Compiled *G Boehm*, Basel
(B) = Book Reviews